Somatic embryogenesis and cryopreservation of cauliflower (*Brassica oleracea* var. *botrytis)*

by

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Copyright statement

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Abstract

Successful efficient whole cauliflower plant regeneration via somatic embryogenesis from root derived callus tissue was achieved. The research confirmed for the first time the capability of mass production of cauliflower somatic embryos through the indirect pathway. The best callus induction and proliferation was on semi solid Murashige and Skoog (MS) medium supplemented with 2, 4-D at 0.15 mg $L^{-}1$ and Kinetin at 0.1 mg L^{-1} and 3% sucrose. The response of different explant types (cotyledon, hypocotyls and root) through callus induction and subsequent culture was determined. The best period for subsequent callus culture was 21 days.

Continuous immersion in agitated liquid medium technique was subsequently used for primary somatic embryo production. The culture requirements were empirically optimized including: explants source and size of callus tissue, blending duration, plant growth regulator combinations and concentrations as well as carbohydrate type and concentration. The highest mean number of somatic embryos (30.9) per explant was achieved using root derived embryogenic callus tissue on MS medium provided with IAA 0.05 mgL^{-1} and Kinetin at 0.5 mgL^{-1} and 2% sucrose. Somatic embryos were developed and matured on this medium and germinated with the highest percentage (60%) on semi-solid MS medium devoid of growth regulators. The culture conditions that led to the formation of secondary somatic embryos were identified. The presence of activated charcoal in the culture medium had an effect on this process but some abnormality of secondary somatic embryos was observed.

Artificial seeds were produced by encapsulating the somatic embryos with a sodium alginate gel (2%) and complexing with calcium chloride (100 mM) for 20 min. The

ability of these artificial seed for germination was evaluated using various combinations of plant growth regulators that were either incorporated in the artificial matrix or in the germination semi-solid culture medium.

It was confirmed that cauliflower root derived embryogenic callus tissue can be cryopreserved following a preculture-dehydration technique. Following cryopreservation, embryogenic cultures can proliferate in agitated liquid medium, and somatic embryos at the globular stage were formed. Also cold storage at 5 °C in the dark was used successfully to store cauliflower callus tissue for three months without diminution of the competence for somatic embryos formation. This ability for cold storage could have a positive effect in reducing costs and efforts that result from subsequent sub-culture. The encapsulation-dehydration technique was assessed for cryopreservation of somatic embryos but failed to lead to survival of any embryos.

Somatic embryos that were produced in this study were able to be well acclimated using a reliable weaning procedure that achieved high rates of survival of plantlets and their subsequent growth to normal plants in the field was assessed. Morphological characteristics of somatic plants compared favourably with zygotic plants but although there was phenotypic similarity, some differences in plant height, curd size and time for curd maturity were observed.

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List of Abbreviations

2, 4-D: 2, 4-dichlorophenoxyacetic acid
ABA: Abscisic Acid
AC: Activated charcoal
ANOVA: Analysis of variances
BA: 6-benzyladenine
$CaCl_2$: Calcium Chloride
CIALMT: Continous immersion in agitated liquid medium technique
CIM: Callus inductiom medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DSE: Direct somatic embryogenesis
ECT: Embryogenic callus tissue
ESEs: Encapsulated somatic embryos
FeEDDHA: Iron ethylenediamine-di *(0*-hydroxyphenyl) acetic acid
FeEGTA : Iron ethyleneglycol-bis (2-aminoethylether) tetraacetic acid
H: Hours
H_3BO_3: Boric acid
HDECT: Hypocotyl derived embryogenic callus tissue
IAA: Indole-3-acetic acid
IBA: Indole-3-butyric acid
ISE: Indirect somatic embryogenesis
Kin: Kinetin
LN: Liquid nitrogen
LSD: Least Significant Difference
MC: Moisture content
min: Minute
MS: Murashige and Skoog
NAA:1- Naphthaleneacetic acid
Na-alginate: Sodium alginate
NAOCL: Sodium hypochlorite
PEG: Polyethylene glycol
PEMs: Pro-embryogenic masses
PGRs: Plant growth regulators
PLBs: Protocorm- like bodies
PVS: Plant vitrification solution
RCBD: Randomized complete block design
RDECT: Root redived embryogenic callus tissue
RNA: Ribonucleic acid
RSE: Recurrent somatic embryogenesis
s: Second
S.E. : Standard error
SEs: Somatic embryos

SIM: Somatic induction medium
SSEs: Secondary somatic embryos
TIALMT: Temprary immersion in agitated liquid medium technique
TIBT: Temporary immersion bioreactor technique

Dedication

I would like to dedicate my thesis to my teacher and my prophet Mohammad

(Peace be upon him)

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Author’s Declaration

At no time during the registration for the degree of Doctor of philosophy has the author been registered for any other University award. I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance whereacknowledged. This study was sponsored by the Iraqi Ministry of Higher Education.

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Chapter one

General Introduction and Literature Review

1.1 Cauliflower plant

Cauliflower, *Brassica oleracea* var.*botrytis*, is an important vegetable crop in the Brassica family (Sharma et al., 2005) and it is one of the most popular vegetables among cole crops (Board, 2004). Its name comes from the Latin word *Caulis* (cabbage) and *Flos* (flower) (Kashyap, 2013). The *Brassicaceae* family contains 3709 species and 338 genera (Warwick et al., 2006) and economically, the genus Brassica can be considered one of the most important in the *Brassicaceae* family (Neeser et al., 1999) since major sources of vegetables, edible oil and condiments are be included in this genus. The two species *Brassica napus* L. and *Brassica rapa* L. are planted mainly for oil production and are characterized as collectively as rapeseed or canola. *Brassica juncea* (L.) Czern & Coss., *Brassica nigra* (L.) Koch. and *Brassica carinata* Braun can play an important role in the production of commercial spices and mustards as either grains or oil (Labana and Gupta, 1993). *Brassica oleracea* L., is more familiar to consumers as the vegetable and fodder crop species and includes cauliflower, cabbage, Brussel-sprouts, broccoli, kale, kohl rabi (Balkaya et al., 2005).

The risk of age-related chronic illnesses such as cardiovascular health and other degenerative diseases as well as several types of cancer can be reduced with a high intake of Brassica vegetables as they contain the anti-oxidative components which are water–soluble and include phenolic compounds (mainly flavonoids), vitamins (mainly ascorbic acid) as well as other anti-oxidant constituents which are lipid-soluble such as carotenoids and tocopherols and glucosinolates. These substances have the potential for reducing physiological and oxidative stress-induced DNA damage. Thus it has been suggested that they have cancer preventive effects and a protective role in other major diseases (Soengas Fernández et al., 2011). Cauliflower

is highly regarded for its nutritional value as it contains high amounts of vitamin C, minerals, antioxidants and soluble fiber (Fernández-León et al., 2012).

Cauliflower is a cross pollinated crop (Sharma et al., 2005) and pollination control mechanisms such as self-incompatibility and male sterility can be applied to encourage cross pollination and the commercial production of hybrid seed (Kucera et al., 2006, Sharma et al., 2005). F1 hybrid seed production is the preferred breeding strategy for Brassica crops yielding highly consistent high quality crops. However the out-breeding nature of these crops creates difficulties in the achievement of pure-bred lines, which are the pre-cursor for F1 hybrid production (Kaul, 1988). Also, the high cost of hybrid seed and relative unavailability have inhibited the popularization of F1 hybrids in many Brassica species (Tripathi and Singh, 2001, Sharma et al., 2005). Thus , the use of a conventional breeding programme in producing hybrids leads to the consumption of much time and resources to obtain and retain the appropriate parental lines (Desai et al., 1997). In cauliflower, there is widespread availability of F1 hybrids for summer and autumn varieties but for winter varieties, which are particularly important for the southwest of England there is still a deficit of available varieties. *In vitro* or plant tissue culture techniques can be used in Brassicas to achieve somatic embryogenesis, organogenesis and regeneration (Antonio et al., 1987, Jain et al., 1988, Ono et al., 1994, Koh and Loh, 2000, Khan et al., 2002). The capability of clonal propagation of Brassica plants *in vitro* is of interest as the plants of this genus tend to be short-lived annuals or biennials (Maheswaran and Williams, 1986b). El-Zeiny (2007) has commented that plant tissue culture techniques can be applied to achieve 10 times more plantlets than those produced from traditional propagation methods. Somatic embryogenesis can also be used to solve several problems, like limited seed availability, and to shorten the long sexual

breeding cycle (Chandrasekhar, 2006). Redenbaugh et al (1986) referred to the difficulties in the production of cauliflower somatic embryos *en-mass* reporting that cauliflower was recalcitrant to somatic embryogenesis. Also, Qin et al., (2006) has stated that the progress of plant propagation in cauliflower as well as somatic embryogenesis was relatively limited.

1.2 Somatic embryogenesis

1.2.1 Definition of somatic embryogenesis

In vitro plant regeneration can occur through organogenesis and somatic embryogenesis, in organogenisis, shoots and roots can form sequentially in response to appropriate culture conditions (often dependent on concentration and type of plant growth regulators) with the presence of vascular connections between mother tissue and the regenerating sections (Jimenez, 2005, Terzi and Lo Schiavo, 1990). Somatic embryogenesis however is the developmental pathway *in vitro* by which bipolar structures that are similar to zygotic embryos are derived from somatic cells without gamete fusion (Emons, 1994, Valdez-Melara et al., 2009, Williams and Maheswaran, 1986c, Raemakers et al., 1995). In asexual embryogenesis (Dodeman et al., 1997) an external bipolar structure is normally formed without connection to the plant or callus vascular system, and it has root and shoot poles (Haccius, 1978). In plant breeding programmes, micropropagation via somatic embryogenesis is frequently described to be more beneficial than organogenesis (Tomar and Gupta, 1988) because subsequent plantlets emanating from a somatic embryo is believed to originate from a single cell. Therefore, plants derived from somatic embryos tend to be similar whereas organs propagate through the collective organization of many cells and may show wide genetic variations (Puhan and Rath, 2012). The word embryoid is frequently used when embryo-like structures appear in

cultures with an appearance similar to zygotic or somatic embryos (George et al., 2008). Somatic embryogenesis is the term that has been used as an important method for multiplication *in vitro* (George et al., 2008). Von Arnold et al (2002) have defined somatic embryogenesis as a pathway in biotechnology and that plant cells, tissue and organs can all form embryos under *in vitro* conditions. The development of a somatic cell into an embryo can be achieved through morphological stages which resemble *in vivo* embryogenesis (Dong and Dunstan, 1999, Gawel, 1989). Basically, both embryos undergo the same stages of development and go through globular, heart shaped, torpedo, cotyledonary and mature embryos morphologies (Pareek, 2005, Gray and Purohit 1991, Xu et al., 1991, Zimmerman, 1993, Mandal and Gupta, 2002). These somatic embryos can also “germinate” and produce new plantlets (Von Arnold et al., 2002) although this is often referred to as “conversion” rather than germination.

In seed plants, embryogenesis can be considered as an important process in producing a new generation. This morphogenetic process involves drastic changes by which a new individual or zygote is generated from a fertilized ovule. The zygote divides asymmetrically and transversely to form a small apical cell and a large basal cell. The apical cell can develop to the embryo proper (Umehara et al., 2007) undergoing a series of complex cellular and morphological processes that finally lead to produce the sporophytic plant (Rao, 1996). The basal cell develops to the suspensor, which remains attached to the mother tissue to provide an “umbilical” through which nutrients and growth regulators are translocated to assist the development of the full embryo (Umehara et al., 2007). The zygote shows some structural and functional characteristics which are intimately linked with the formation of the first embryonic developmental stages. These features can be utilized as points

of reference to better understand the initiation of somatic embryogenesis (Dodeman et al., 1997).

In the somatic embryogenensis process, either haploid or diploid cells can regenerate complete plants through histodifferentiation patterns that are analogous to zygotic embryos (Williams and Maheswaran, 1986) through a series of morphological and biochemical changes that lead to the production of a bipolar structure without vascular connection with the original tissue (Quiroz-Figueroa et al., 2006) and without the participation of sexual organs and cells (Umehara et al., 2007) (Fig.1).

George el al (2008) mentioned that the first observation of somatic embryo formation was in Carrot (*Daucus carot*a) cell suspensions by Steward et al (1958) and Reinert (1958) and since then, somatic embryogenesis has been reported from a large number of plant species. Plant regeneration via somatic embryogenesis can be achieved through five steps (George et al., 2008):

Firstly, initiation of embryogenic cultures from the primary explant on medium that contains plant growth regulators (PGRs) such as Auxin and Cytokinin.

Secondly, proliferation of embryogenic cultures (Von Arnold 1996) on medium supplemented with PGRs similar to initiation.

Thirdly, pre-maturation of somatic embryo on medium without PGRs to inhibit regeneration and induce somatic embryo formation,

Fourthly, maturation by culturing on medium with ABA.

Fifthly the regeneration of plants from somatic embryos on medium lacking PGRs (George et al., 2008).

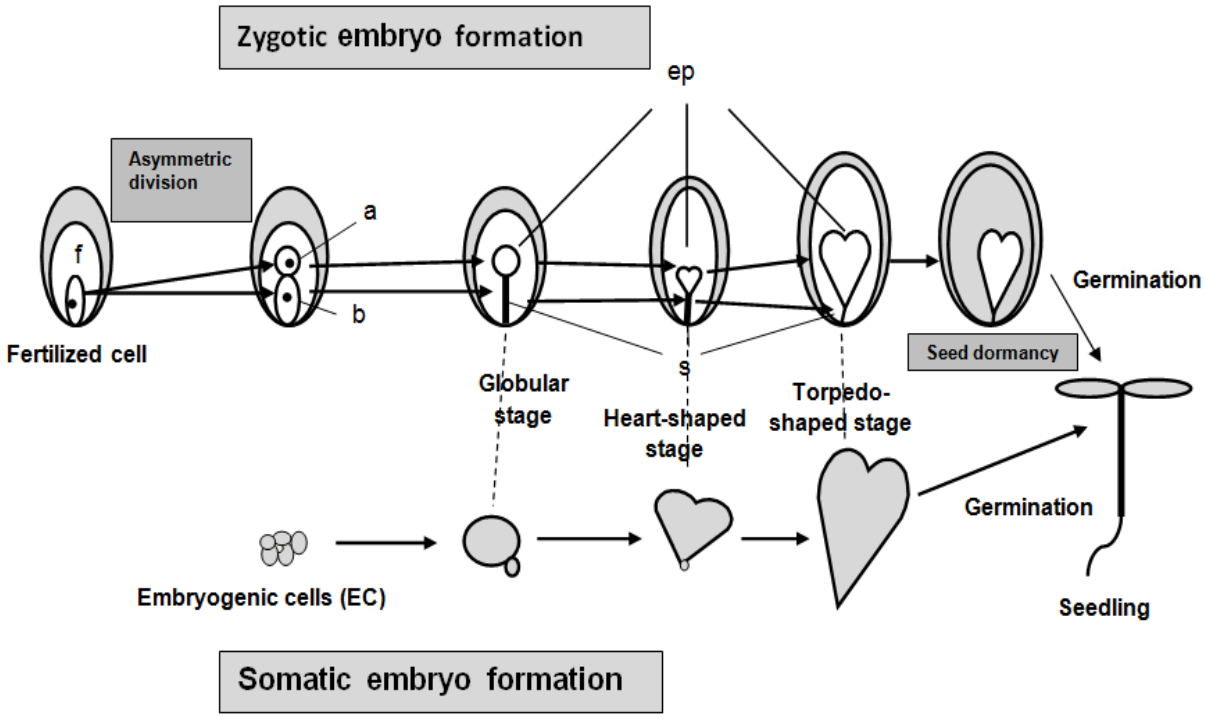


Figure 1. Model scheme shows zygotic and somatic embryo formation in angiosperms. Somatic embryogenesis is morphologically and developmentally analogous to zygotic embryogenesis in both temporal and spatial aspects: a, apical cell; b, basal cell; ep, embryo proper; f, fertilized egg; s, suspensor (modified from Umehara et al., 2007).

1.2.2 Pathways of somatic embryogenesis

Somatic embryogenesis has been obtained by two pathways: direct somatic embryogenesis (DSE) or indirect somatic embryos (ISE) (Jimenez, 2005, George, 1993, Puhan and Rath, 2012, Slater et al., 2003).

1.2.2.1 Direct somatic embryogenesis

The induction of somatic embryos can be achieved directly from organized tissue (Slater et al., 2003) of the stem, leaf, microspores or protoplasts without embryogenic calli proliferation (Jimenez, 2005). The formation of DSEs requires the presence of an exogenous growth regulator or favorable conditions to develop (Williams and Maheswaran, 1986, Wann, 1988, Evans et al., 1981).

1.2.2.2 Indirect somatic embryogenesis

Somatic embryos can be obtained indirectly through callus formation in *in-vitro* tissue culture (Williams and Maheswaran, 1986). This pathway includes dedifferentiation of organized tissue into callus tissue before embryo formation (Slater et al., 2003). The induction phase is required for cells to acquire embryogenetic competence because the somatic cells are not naturally embryogenetic (Namasivayam, 2007). Callus induction can also be applied as a major way to generate somaclonal variation, and it is one of the most important steps for genetic transformation research (Alam, 2002). The use of different explants such as root, hypocotyl and cotyledon can be applied for callus production in Brassica seedlings (Fuller and Fuller, 1995). Lashari et al. (2008) have found that callus can be classified into two kinds-embryonic and non-embryogenic callus. Embryogenic callus development into different stages of somatic embryos. Deane et al., (1997) have described that callus tissue of cauliflower consists of two types of cells, yellow embryogenic cells and green non-embryogenic cells and they referred that the green non-embryogenic cells might be provide crucial factors for somatic embryo-like structure formation, however Deane et al (1997) failed to report reliable ISE production in cauliflower. Chamandosti et al.,(2006) have reported that three morphological types of calli can be distinguished from hypocotyls explants of canola (*Brassica napus* L.) yellow calli, somatic embryos were observed from this type and white calli which was organogenic (shoots were developed from these calli). The third callus type was dark brown and did not differentiate and died.

The visual distinction between embryogenic and non-embryogenic callus is easy and depends on morphology and color. Embryogenic callus is also commonly described as containing pro-embryogenic masses (PEMs) (George et al., 2008). These can be

characterized by their yellow color and their globular structure and contrasts with the wet aspect, translucence, and more brownish colour of non embryogenic callus (Van Sint Jan et al., 1990) cited in (Gandonou et al., 2005). Craig et al., (1997) have reported that the somatic embryogenic callus that promotes globular embryo-like structures was a compact nodular callus in Purple Mistress (*Moricandia arvensis*). Also, embryogenic callus can be friable as was reported by Ganapathi et al., (2001), Fki et al.,(2003) and Chithra et al., (2005) for banana cv. Rasthali (Musa spp. AAB group) , date palm *Phoenix dactylifera* L., cv. Deglet Nour and the woody medicinal plant *Rotula Aquatica* Lour. respectively. Somatic embryos can develop from this friable embryogenic calli (Jimenez, 2005).

Embryogenic cells are characterized as unique cells, superficially they are similar to meristematic cells but, generally they are more isodiametric in shape, smaller, have larger, more densely staining nuclei and nucleoli, and have a denser cytoplasm (Williams and Maheswaran, 1986, Carman, 1990).

Somatic embryos that form via direct or indirect somatic embryogenesis could have initiated from either a single cell (unicellular origin) or from a group of cells (multicellular origin) (Williams and Maheswaran, 1986, Yeung, 1995, Quiroz-Figueroa et al., 2006). When somatic embryos had a unicellular origin, only a single cell of the epidermal layer of explant tissue actively divides for initiation and a basal part that becomes a suspensor-like structure that makes the connection between the somatic embryo and the maternal tissue (Williams and Maheswaran, 1986, Quiroz-Figueroa et al., 2006). For the multicellular origin, fusion of the basal region might occur directly to their maternal tissue without formation to a suspensor-like structure (Wannarat, 2009). The morphological changes during somatic embryogenesis were reported in a Chinese medicinal plant by Gui et al (1991) who observed that active

cell division in a group of epidermal cells at the callus surface was the first sign of somatic embryogenesis. After that, tiny cylindrical protrusions developed from these active cells and became globular-shaped embryos that then developed into cotyledon shaped embryos.

1.2.3 Morphology of zygotic and somatic embryos

There are two types of embryogenesis in plants: zygotic and somatic. somatic embryogenesis is a process by which somatic cells can be differentiated to somatic embryos (Joshi and Kumar, 2013). Somatic cells require the signal for the cell polarization and the asymmetric division given by auxins as it happens in their zygotic counterparts (Gutiérrez-Mora et al., 2004, Pagnussat et al., 2009). The competent cells are representing an intermediate state between somatic and embryogenic cells. Cellular competence is associated with the dedifferentiation of somatic cells that allows them to respond to new developmental signals. It is well accepted that embryogenic competent cells are morphologically recognized as small, rounded cells with rich cytoplasm and small vacuoles. Thus, they are very similar to meristematic cells or zygotes and this similarity can be further emphasized by their asymmetric division (Fehér, 2005). The morphological and physiological characteristics of somatic embryos are analogous to those of zygotic embryos (Ikeda and Kamada, 2006). The development of zygotic embryo can be classified into four general sequential stages of morphogenetic change: globular-shaped, heart-shaped, torpedo shaped and cotyledonal stages in dicots (Goldberg et al., 1994). In dicotyledonous somatic embryogenesis, small globular embryos initially form which then undergo isodiametric growth and establish bilateral symmetry. These then can develop into the heart stage embryo in which both cotyledons and root and shoot meristems are clearly established. After that, the development proceeds with the

formation of torpedo and subsequently plantlet stages. The plantlets consist of green cotyledons, elongated hypocotyls and developed radicals with very fine root hairs (Zimmerman, 1993). On the other hand, several authors have emphasized morphological and histological differences between somatic and zygotic embryos at corresponding stages of development (Čellárová et al., 1992, Erdelska and Sýkorová, 1997). However, some differences in origin, development and morphology between somatic and zygotic embryos that can be observed might have been owing to the culture conditions of *in vitro*, and genetic changes in the plant material cannot be ruled out, especially when the somatic embryos form with an intervening callus phase (Fras et al., 2008).

1.2.4 Morphological characteristics of somatic and zygotic plantlets *in vivo*.

It was emphasized that phenotypic evaluation should not be neglected as a tool can be used to assess the genetic integrity of the somatic embryogenesis process (Tremblay et al., 1999). However, the similarity in shoot and root morphology for interior spruce [*Picea glauca* (Moench) Vos × *Picea engelmannii* Parry] platelets derived from somatic embryos and from the seeds was reported by Webster et al. (1990). The quality of *Coffea arabica* L. somatic plantlets that produced under *in vivo* conditions was reflected in better growth of the aerial and root systems as well as by similar morphological characteristics to seedlings (Barry-Etienne et al., 2002). Furthermore, white spruce (*Picea glauca*) somatic plantlets established in the greenhouse were similar to control plantlets obtained from germinated seeds (Tremblay, 1990). Also, Celestino et al., (2009) stated that the growth of cork oak *(Quercus suber)* plants obtained from somatic embryos likes seedlings in a field and no signs of abnormal growth were detected, and all plants seem to follow the same growth patterns. Moreover, no abnormalities were observed in pecan trees [*Carya*

illinoinensis (Wangenh.) C. Koch] that regenerated from somatic embryogenic cultures when grown in the field for 4 years (Vendrame et al., 2000).

1.2.5 Somatic embryogenesis in Brassica

1.2.5.1 Direct somatic embryogenesis in Brassica

Somatic embryogenesis has been induced directly in the Brassica genus such as: in *Brassica napus*, direct somatic embryos were obtained from mesophyll protoplasts isolated from androgenetic plants of two cultivars ("Loras" and "Tower") (Li and Kohlenbach, 1982). Somatic embryos were formed directly from the swollen hypocotyl surfaces of late torpedo to the early cotyledonary–stage of zygotic embryos of *B. napus* (Pretova and Williams, 1986). Somatic embryogenesis in rapid-cycling *B. napus* was induced directly from hypocotyls and cotyledons of immature zygotic embryos on MS basal medium after 2-3 subcultures onto the same culture medium (Koh and Loh, 2000). However, somatic embryos were produced directly from immature seeds of double haploid lines of spring *B. napus* on medium free of plant growth regulator (Burbulis and Kupriene, 2005, Burbulis et al., 2007).

In *Brassica juncea,* isolated mesophyll protoplasts could directly produce somatic embryos on medium including an auxin and a cytokinin (Eapen et al., 1989). After that, haploid embryos formed through anther culture of *B. juncea* appeared to have a high ability for direct formation of somatic embryos (Prabhudesai and Bhaskaran, 1993). The induction, growth and development of Indian mustard (*Brassica juncea* L. Czern & Coss) var. RLM 198 somatic embryos was achieved from hypocotyl explants (Kumari et al., 2000).

In *Brassica campestris,* direct somatic embryoids were obtained from superficial cells of the lower hypocotyl of immature zygotic embryos (Maheswaran and Willams, 1986b). Choi et al., (1998) have reported in *B. campestris* the formation of direct somatic embryogegesis from 17% of ovules on MS medium containing *2*,4-D 1 mgL^{-1}, also somatic embryos were produced from cotyledonary explants of immature zygotic embryos on MS medium containing *2*,4-D.

In *Brassica nigra* embryogenesis has also been achieved from seedling–derived hypocotyl explants of four genotypes of *B. nigra* and embryos produced could germinate/convert into seedlings (Narasimhulu et al., 1992).

In *B. oleracea,* direct initiation of somatic embryos in broccoli (*B. oleracea* L. var. *italica*) was observed from the pericycle cell layers of root explants when they were cultured in MS liquid medium containing 1 mg L^{-1} *2*, 4-D, the increase in *2*,4-D concentration led to an increase in the number of normally developed somatic embryos (Yang et al., 2010). Pavlovic et al., (2012) also reported that they produced somatic embryos directly from immature zygotic embryos at the cotyledonary stage of both cabbage and cauliflower.

1.2.5.2 Indirect somatic embryogenesis in Brassica

Indirect somatic embryogenesis in *B. napus* was achieved with a limited number of plants which were obtained from cultures of isolated protoplasts from stem embryos (Kohlenbach et al., 1982). The mesophyll protoplasts of *B. napus* cv. Loras and Tower were used to produce proembryos from microcalli on medium containing *2*,4-D (0.2 mg L^{-1}) and Kinetin (3.0 mg L^{-1}), when the medium was replaced with a medium containing a lower concentration of *2*,4-D, more somatic embryos were achieved (Li and Kohlenbach, 1982). Efficient induction of somatic embryogenesis from hypocotyls protoplast–derived calli of two rapeseed (*B. napus* L.) varieties

‘Ceres’ and ‘Duplo’ was achieved using a system of agarose plating and agarose bead culture by Kirti (1988). The formation of somatic embryos via microcalli was described by Simmonds et al (1991) in *B. napus* cv. Jet Neuf protoplasts isolated from suspension culture at a low density culture technique. Somatic embryos have also been reported from embryogenic calli of *B. napus L*. using a simple procedure for somatic embryogenesis (Majd et al., 2006, Chamandosti et al., 2006). Thereafter, the initiation and development of canola (*B. napus* L.) somatic embryos from hypocotyls-derived callus were investigated at a low concentration of sucrose (20%) (Ahmad et al., 2008). Also in *B. napus* species, two genotypes (Talayeh and RGS003) and the explants segment (hypocotyls and cotyledon) were applied in *in vitro* conditions to produce somatic embryos, the Talayeh genotype produced more somatic embryos and the hypocotyls were more suitable for somatic embryogenesis (Zeynali et al., 2010).

In *Brassica juncea*, hypocotyls segments were used to induce and develop indirect somatic embryos in a rapid single–step method, normal flowering and seed setting were achieved from the propagated plants (Kirti and Chopra., 1989). Kirti and Chopra (1990) also described a procedure to induce somatic embryogenesis from microcalli obtained from protoplasts derived from hypocotyls of *B. juncea* seedlings. Several hundred propagated plants using this procedure were acclimated in a growth chamber and subsequently transferred to soil. Indirect somatic embryogenesis from hypocotyls and cotyledons with a high frequency was also established in mustard (*B. juncea* L. cv. Pusa Jai Kisan) (Akmal et al., 2011).

In *Brassica campestris*, indirect somatic embryogenesis was produced from callus tissues derived from petiole explants (Bhattacharya and Sen, 1980) and from cotyledonary explants of mature zygotic embryos via callus formation (Choi et al.,

1996a). Fan et al (2006) have observed the formation of somatic embryos from protoplast of *B. campestris ssp*, *pekinensis* on calli of the genotype Asko in shoot-inducing medium including 3 mg L^{-1} $AgNO_3$ and the embryos easily gave rise to intact plants.

In *Brassica nigra* (L.) Koch (black mustard), somatic embryos were obtained from protoplast-derived calli isolated from cell suspensions that induced from thin cell layer explant-derived calli. Somatic embryos were initiated in liquid medium supplemented with 2, 4-D, NAA and BA and in medium devoid of growth regulators (Klimaszewska and Keller, 1986). Gupta et al., (1990) have referred to a method that can be followed to produce plants from embryogenic callus of *B. nigra* through somatic embryogenesis and the morphogenic ability of embryogenic callus which is maintained even after 12 subcultures. After that, the regeneration through somatic embryogenesis was obtained from unorganized callus tissue that was derived from hypocotyls and root explants of *B. nigra* seedlings (Mehta et al., 1993).

In *Brassica oleracea,* Pareek and Chandra (1978) have reported somatic embryogenesis from leaf callus of cauliflower (*B. oleracea* var. *botrytis)* on MS medium supplemented with IAA (1 mg L^{-1}) and Kin (0.5 mg L^{-1}). Somatic embryos were achieved after transferring callus tissue to medium with low concentration of IAA (0.01-0.1 mg L^{-1}) but failed to proliferate. However, protoplasts were isolated enzymatically from the first leaves of cabbage (*B. oleracea* var *capitata*, F1 hybrid 'Baochun'). Somatic embryogenesis was achieved from small calli and formation of plants was identified (Fu et al., 1985). Embryogenic calli derived from hypocotyl protoplasts of *B. oleracea* were reported to generate somatic embryos when were cultivated in the complete absence of 2, 4-D, and SEs were easily distinguished by red cotyledons owing to the presence of anthocyanin. Decreasing numbesr of SEs

were reported when 2,4-D was present in the medium (Fransz et al., 1993). Somatic embryo-like structures were obtained from leaf derived - yellow callus tissue of cauliflower by Deane et al., (1997) and have referred that the production of embryogenic callus tissue and somatic embryo-like structures were affected by culture vessel type. Jars vessels were better than petri dishes possibly because greater aeration was provided to the cultures using this type of vessel. Similarly, Leroy et al (2000) have reported that 1-3 week old hypocotyl segments of cauliflower could produce bright-green callus tissues when were cultured in medium consisting of 2, 4-D (1 mg L^{-1}) and Kinetin (1 mg L^{-1}) plus 3% sucrose. More than a quarter (27%) of the callus tissues became embryogenic when subcultured on the same medium with a reduced concentration of sucrose to 2%. Somatic embryogenic callus has also been reported to be initiated from hypocotyl explants of cauliflower after the development of explants occurred by adding sucrose at 3% in MS medium supplemented with 2,4-D (1 mg L^{-1}) and kinetin (0.1 mg L^{-1}) (Raut, 2003). In *B. oleracea* L. var. *italica* subvar. Cymosa, the use of stigmas with style from broccoli pistils led to the production of indirect somatic embryogenesis on Murashige and Skoog basal medium (Zenkteler et al., 2006). However, high efficiency system for somatic embryogenesis and shoot proliferation of broccoli (*B. oleracea* L. var. *italica*) via callus induction has also been reported (Qin et al., 2007).

Direct and indirect somatic embryogenesis was established with a high efficiency from immature cotyledons of *B. napus* cv.'Topas' on MS medium supplemented with different concentration of NAA and BA (Turgut et al., 1998). Also, somatic embryos of *B. napus L*. Were identified directly and indirectly from canola cultivars grown under the Egyptian agricultural conditions (Moghaieb et al., 2006).

In cauliflower, direct and indirect initiation of somatic embryogenesis was investigated in culture of 2-week-old hypocotyl explants on MS medium supplemented with 1.0 mg L^{-1} 2,4-D and 0.5, 1.0 and 1.5 mg L^{-1} Kinetin (Siong et al., 2011). Despite this recent report, the lack of publications in the literature concerning cauliflower somatic embryogenesis is testimony to its recalcitrant nature as defined by Redenbaugh et al 25 years ago. Thus, whilst somatic embryogenesis is rarely reported in cauliflower it is reported in closely related sister species.

1.2.6 Factors affecting somatic embryogenesis:-

Many studies have reported the effect of environmental conditions on culture induction, maintenance, somatic embryogenesis and plant regeneration (Zhang and Li, 1995, Hi and Li, 1998). Also many factors associated with *in-vitro* culture can affect the efficiency of regeneration such as medium composition, growth regulator type and concentration, culture age and explant genotype and its pre-culture growing environment (Gawel, 1989, Corredoira et al., 2003, Gonzalez et al., 2001, Jonoubi et al., 2004, Akasaka-Kennedy et al., 2005, Kamal et al., 2007, Ben Ghnaya et al., 2008). Generally, it has been thought that the modifications of different exogenous and endogenous factors including growth regulators can lead to somatic embryogenesis (Steward et al., 1964). The developmental stage of the explant tissue has determined the requirement for PGRs used for the initiation of somatic embryos. Usually, synthetic auxins are added in media to induce embryogenic callus formation and embryogenic culture initiation (Kutschera, 1994). Somatic cells can be triggered to differentiate into embryogenic competent cells using both auxins and cytokinins (Feher et al., 2003). Carbohydrates have also been reported to be important in somatic embryogenesis and embryo culture. Sucrose is the most common carbohydrate which is used as an energy source in tissue culture but

somatic embryogenesis can be induced by other carbohydrates and high numbers of embryos can be achieved by using the carbohydrates glucose or lactose (Ricci et al., 2002). Among carbohydrates, sucrose is preferred in several species that are propagated in tissue culture for induction, regeneration and maturation of embryos (Norggard, 1997, Ainsley and Aryan, 1998). The successful induction of somatic embryogenesis in plants can usually be achieved using sucrose as the source of carbon (Custers et al., 1988, Chee, 1990, Cade et al., 1990).

1.2.7 Application of somatic embryogenesis

Somatic embryogenesis is very valuable and can be applied to achieve a wide range of objectives, from basic biochemical, morphological or physiological studies, and the improvement of technologies with a high degree of practicable application to agriculture and horticulture (Jimenez, 2001). Studies of somatic embryogenesis are very important due to having both scientific and applied aspects (Von Arnold et al., 2002). One of the main uses of somatic embryogenesis is employment to investigate the initial events of zygotic embryogenesis in higher plants. There is still limited progress in the understanding of the developmental events in plant embryos and zygotic embryos of higher plants consist of several tiny cells that grow within maternal tissue, such as immature fruits or flowers and thus it is difficult to collect sufficient embryos for analyses of the biological events that occur early in the developmental process. Somatic embryos can provide a good model system by which such problems are circumvented in a number of plant species (De Jong et al., 1993, Kiyosue et al., 1993, Zimmerman, 1993) since the developmental stages of somatic embryos resemble those occurring in zygotic embryogenesis (Dodeman et al., 1997). Somatic embryogenesis is also a benefit for mass propagation of clonal materials (Hartmann et al., 1997) and is a significant method used for plant

regeneration to develop genetically uniform plants (Gatica-Arias et al., 2008, Etienne et al., 2002, Thomas et al., 2004, Thakare et al., 2008, Lee et al., 2003). The establishment of an ideal somatic embryogenesis procedure is an essential prerequisite for production of a successful artificial seed technology (Buyukalaca and Mavituna, 1995, Latif et al., 2007, Vicient and Martinez, 1998, Nakagawa et al., 2001, Maruyama et al., 2003, Jain and Gupta, 2005) that can be directly cultivated into the field (Redenbaugh, 1993). Clonal propagation can also be obtained by somatic embryo culture and germplasm conservation through somatic embryogenesis (Maruyama et al., 2003) for establishment of gene banks (Von Arnold et al., 2002). Somatic embryos have another advantage for plant proliferation as they can be used to scale-up plant quantities using bioreactors to produce unlimited numbers of somatic embryos that have the ability to germinate and convert to complete plants (Von Arnold et al., 2002). Reduction in labor costs and production of a high percentage of uniform embryos could be achieved using bioreactors (Ibaraki and Kurata, 2001). The other application for somatic embryos is secondary somatic embryo producation that can be used for increasing the yield of propagated plants (George et al., 2008). Secondary embryogenesis, or sometimes termed recurrent, repetitive, accessory or proliferative embryogenesis has occurred when primary somatic embryos cannot develop to plantlets and second generation embryos can develop from cotyledons, hypocotyls or roots of primary embryos to form secondary embryos (Maheswaran and Williams, 1986a, Maheswaran and Williams, 1986b, Kato, 1989, Polito et al., 1989, Plata and Vieitez, 1990, Vieitez and Barciela, 1990, Gui et al., 1991, Tenning et al., 1992). Recurrent somatic embryogenesis (RSE) has been reported to be a useful method to achieve genetic transformation of plants (McGranahan et al., 1988, McGranahan et al., 1990) and it

can also be applied for the production of artificial seed (McKersie et al., 1989, Slade et al., 1989) since unlimited numbers of somatic embryos can be provided by the RSE system (Parrott and Bailey, 1993). The phenomenon of secondary embryogenesis in *Brassica* species can be applied for embryo cloning and rapid proliferation of novel genotypes or mutants (Ingram et al., 1984, Palmer et al., 1996).

1.2.8 Limitation of somatic embryogenesis

Although somatic embryogenesis can offer great potential, it has some limitations such as, firstly, asynchronous development of somatic embryos (Zimmerman, 1993, Zegzouti et al., 2001) and therefore, in one culture system all stages of embryos can be observed (Deo et al., 2010). Secondly, the stability of cell lines. After a period of time, the regeneration might become impossible as the proportion of cells that complete embryogenesis will decrease. Also, prolonged time in culture can lead to accumulation of mutations (somaclonal variations), which can cause morphological abnormalities such as fused cotyledons, pluricotyledony and formation of multiplex apex (Evans et al., 1983). Thus, the initiation of new culture is prequisite for a stable system as the old one might lose regenerability (Deo et al., 2010).

1.3 Artificial seeds

1.3.1 Definition of artificial seeds

Artificial seeds or synthetic seeds are defined as somatic embryos engineered to be used in commercial plant production (Gray and Purohit 1991, Redenbaugh, 1993) and somatic embryogenesis is often a model system used to produce synthetic seed (Philips and Gamborg, 2005, Latif et al., 2007). Artificial seeds, often called clonal seeds, somatic seeds, somseeds, synthetic seeds or synseeds, could be an alternative to true seeds as they may include somatic embryos that are covered by an artificial coat. This definition depends on the similarity of somatic embryos with

zygotic embryos in physiology, morphology and biochemistry (Redenbaugh et al., 1986, Redenbaugh et al., 1988). The first form of synthetic seed was produced using hydrated somatic embryos, but the labour involved led to high costs and the propagules were very delicate. The development of alginate capsules that encapsulated a single embryo in a protective coating partially avoided this problem and is now used routinely in such work (Redenbaugh et al., 1986, Fujii et al., 1989, Fujii et al., 1992) (Fig. 2). The encapsulation technology can be considered as a promising approach which can be used for exchange of plant materials between both public and private plant tissue culture laboratories. Also to achieve germplasm conservation, the propagules derived from *in vitro* culture or by micropropagation can be applied directly in nurseries or in a field (Standardi and Micheli, 2013). The technology of artificial seeds has been developed to use somatic embryos and/or other micropropagules like axillary shoot, buds and apical shoot tips (Ara et al., 2000, Ravi and Anand, 2012) or stem and root segments (Vdovitchenko and Kuzovkina, 2011). By using the benefits of a vegetative regeneration system with the capability of long-term storage, different applications of synthetic seeds in agriculture have been made (Gray and Purohit 1991, Redenbaugh et al., 1991, Redenbaugh, 1993, Ara et al., 2000). Thus, new vistas in agriculture have been opened up using artificial seeds that have been developed from somatic embryos and non zygotic tissues for plant propagation (Saiprasad, 2001). Crops which are useful for artificial seed production can be classified into two categories:

1) Those that have a strong technological basis, high quality of somatic embryos are produced from this type;

2) Those with a strong commercial basis (Redenbaugh et al., 1987).

The encapsulation of somatic embryos is successfully applied to prepare the artificial seed in several plant species like sandalwood (Bapat and Rao, 1988),*Citrus reticulate* (Antonietta et al., 1998), *Hopea parvitflora* (Sunilkumar et al., 2000), *Paulownia elongate* (Ipekei and Gozukirmizi, 2003), sugarcane (Nieves et al., 2003), *Daucus carota* (Latif et al.,2007), *Pinus radiata* (Aquea et al., 2008), *Nothofagus alpina* (Cartes et al., 2009) and *Catharanthus roseus* (L.) G. Don (Maqsood et al., 2012). Redenbaugh *et al.* (1986) reported using somatic embryos (SE) of cauliflower which had been encapsulated as single-embryo beads to produce artificial seeds.

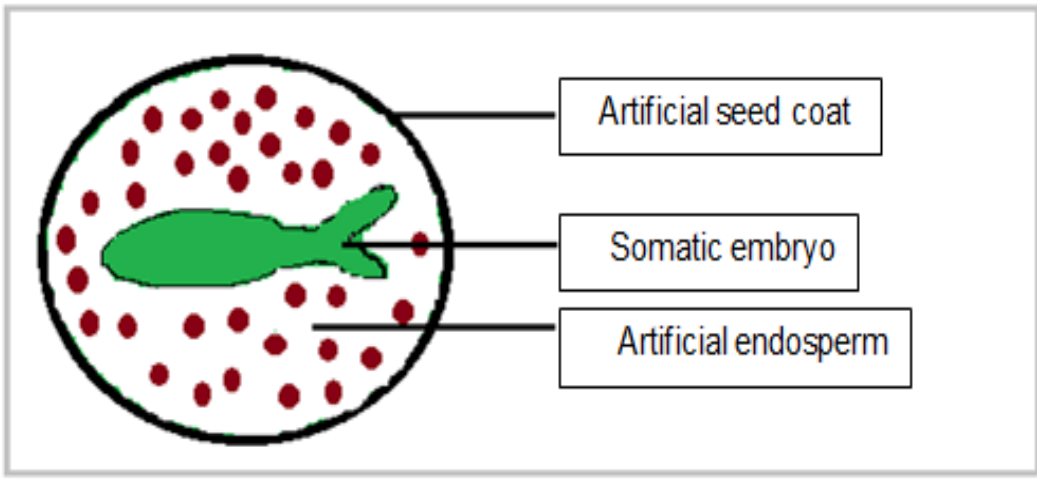


Figure 2. Artificial seed concept (modified from Saiprasad, 2001).

1.3.2 Types of artificial seeds:-

Two types of artificial seeds are commonly produced: desiccated and hydrated (Ara et al., 2000, Jain and Gupta, 2005).

1.3.2.1 Desiccated artificial seeds

Desiccated artificial seeds are achieved from somatic embryos either naked or encapsulated in polyoxyethylene glycol followed by their desiccation. Desiccation

can be applied either rapidly by leaving in unsealed petridishes on the bench overnight to dry, or slowly over a more controlled period of reducing relative humidity (Ara et al., 2000).

1.3.2.2 Hydrated artificial seeds

Hydrated artificial seeds can be produced by encapsulating somatic embryos in hydro-gel capsules. They are produced in plant species which are recalcitrant and sensitive to desiccation (Ara et al., 2000). Encapsulation is expected to be the best method to supply protection and to convert the *in vitro* micropropagules into 'artificial seeds' or 'synthetic seeds' or 'synseeds' (Redenbaugh, 1993) and is an important application of micropropagation to develop the success of *in vitro* derived plant delivery to the field. However, somatic embryos need to be encapsulated in a suitable material that promotes germination (Latif et al., 2007). Previously, several gelling agents such as, polyco 2133 polyox, agar, agarose, alginate, gelrite, carboxy methylcellulose, carrageenan, guar gum, tragacanth gum polyacrylamide, sodium pectate ethylocellulose and nitrocellulose have been tested to produce artificial seeds (Ara et al., 2000, Saiprasad, 2001, Lambardi et al., 2006). Alginate or similar products are now used routinely to maintain the hydration of the embryo and can contain nutrients and growth supplements (Shargool and Ngo, 1994) and the most popular is sodium alginate. For alginate encapsulation the somatic embryos are mixed with sodium alginate gel (0.5-5.0% w/v) and dropped into a calcium salt solution and sodium ions are replaced by calcium ions and capsules form surrounding the somatic embryos (Ara et al., 1999, Redenbaugh, 1993, Redenbaugh and Walker, 1990). The ability of Na-alginate solution for long storage and to mix with a nutritive medium to produce synthetic endosperm as well as the rigidity of alginate beads can supply somatic embryos with an effective protection. However,

the alginate has some unfavorable characteristics such as the “tacky” or sticky nature of the beads. The coats of alginate artificial seed have a sticky surface and they are very moist, thus the seeds adhere to each other and not easy to separate. The rapid dehydration of the alginate beads which become hard in just a few hours after exposure to air can lead to difficulties or prevent the conversion to plantlets and this is considered another limitation (Redenbaugh et al., 1988). The necessity of a hydrophobic layer at the surface of the artificial seeds which avoids adhesion for beads and prevents loss of water was first understood by Redenbaugh et al., (1987) but has been difficult to realize.

1.3.3 Uses of artificial seeds

Zygotic embryos are formed from the sexual recombination of male and female gametes therefore, in many species genetic variability occurs which are minimal in the seeds of self–pollinated crops but are substantial in cross-pollinated species. Also, some important crop species, are sterile and do not set viable seed, thus, cuttings or other vegetative means are used for propagation and these methods and these rarely present convenient storage solutions. Furthermore, seeds may be infected with pathogens which can spread from contaminated seed to production fields. Synthetic seeds can be applied to overcome these limitations and achieve the clonal regeneration of large numbers of disease–free propagules (Shargool and Ngo, 1994). Artificial seeds can also be used in the propagation of male (or female) sterile plants for hybrid seed production (Saiprasad, 2001). Artificial seeds produced through somatic embryos can be a useful technique for transgenic plants where a single gene can be placed in a somatic cell and then this gene will be located in all the plants which are produced from this cell. Therefore, artificial seeds could be an efficient technology which can be applied for the reproduction of transgenic plants

(Daud et al., 2008). Artificial seeds provide a protective coating to an *in vitro* propagule and increase the level of micropropagules success in the field by reducing the stresses from drought and pathogens under natural environmental conditions (Ara et al., 2000)

1.3.4. Limitations of artificial seeds

Although there are a lot of advantages for artificial seeds technology there are still some limitations such as,

Limitation in storage duration caused by lack of dormancy (Reddy et al., 2012).

Synchronic deficiency in somatic embryo development (Reddy et al., 2012).

Improper maturation and low level of conversion into plantlets (Ara et al., 2000c, Reddy et al., 2012).

Limitation in production of viable mature somatic embryos (Reddy et al., 2012).

Viability and plant recovery is often reduced when the artificial seeds are stored at low temperature (Makowczyńska and Andrzejewska-Golec, 2006).

1.4 Cryopreservation

1.4.1 Definition of cryopreservation

Cryopreservation is described as the most valuable method used for long-term germplasm conservation. Cryopreserved materials require limited space, low maintenance and are protected from contamination (Scocchi et al., 2004). The storage of viable cells, organs tissues and organisms at ultra-low temperature, usually in liquid nitrogen to a minimum temperature of -196^{o}C (Benson, 2008) can be really beneficial especially in gene banks. At this temperature there is cessation of biological activities and materials can be stored for extremely long periods (Grout, 1995). Under the correct conditions plant materials can be stored without

modification or deterioration for an indefinite period of time (Lambardi et al., 2000, Mannonen et al., 1990). The conservation of plant genetic resources *in vitro* has become a complementary pathway to the conventional conservation methods (Shibli et al., 2006). In this preservation strategy, different plant materials can be used such as shoot tips, cell cultures, embryos and seeds (Feng et al., 2011).

The storage of embryogenic calluses in liquid nitrogen is applied to preserve their regeneration capacity (Anandarajah et al., 1991) and to reduce the risks of somaclonal variation which increase with culture duration and frequency of sub-culturing (Martinez-Montero et al., 1998). The development of cryopreservation methods of somatic embryos has been investigated as a next progress step to artificial seed technology (Tessereau et al., 1994).

1.4.2 Theoretical basis of cryopreservation

The use of cryopreservation for biological tissues can be successful only if intra–cellular ice crystal formation, which causes damage to the cells, is prevented. Ice crystal formation at sub-zero temperatures can be avoided or minimised through the synthesis of specific substances such as sugars, proteins and proline that lower the freezing-point in living plant cells, resulting in avoidance of crystallization and maintaining a minimal moisture level needed to maintain viability (Sakai, 2000).

1.4.3 Cryopreservation of somatic embryos

Different techniques are published describing cryopreservation of somatic embryos such as vitrification, encapsulation-dehydration, pre-growth, pre-growth desiccation, desiccation (Sharma, 2005) as well as encapsulation-vitrification and droplet-vitrification (Engelmann, 2011).

1.4.3.1 Vitrification

Vitrification is a simplified cryopreservation procedure that includes rapid freezing through direct immersion in LN (Sakai et al., 1990, Grout, 1995, Towill, 1995) Vitrification can be followed to prevent ice crystal formation (Gonzalez-Arnao et al., 1996, Dumet et al., 1993c) without an extreme reduction of cellular water. Vitrificationin in this context refers to the physical process of transition of an aqueous solution into an amorphous and glassy state at sub-zero temperatures where ice crystals are absent (Sakai, 2000, 2007).

In this process, the explants are treated with loading solutions such as MS + 2.0 M glycerol + 0. 4 M sucrose for 20 min at room temperature, followed by treatment with a plant vitrification solution [PVS2; MS+30% glycerol + 15% ethyleneglycol + 15% DMSO (dimethyl sulfoxide) + 0.4M sucrose] at room temperature or at 0^{o}C (Sharma, 2005, Sakai and Engelmann, 2007). Other plant vitrification solution can be used such as PVS3 which consists of 40% (w/v) glycerol and 40% (w/v) sucrose in basal culture medium (Sakai and Engelmann, 2007). The explants are then frozen rapidly in LN, followed by rapid thawing and treatment with unloading solution (MS + 1.2 M sucrose) for 20 min (Sharma, 2005, Sakai and Engelmann, 2007). Thawing is usually rapid using a water bath at 40^{0}C to avoid crystallization and ice crystal growth which can occur during slow warming and produce intracellular damage. Thawing is followed with material produced *in vitro* such as somatic embryos which still have high levels of water (Withers, 1979). However, the chemical toxicity or osmotic stress that resultes from exposure to the vitrification solution can cause damage to plants. Therefore, careful vitrification solution exposure is critical (Sakai et al., 2000). This technique has already been applied to preserve a large number of species (Sakai et al., 2008).

Sakai and Engelmann, (2007) reported that the vitrification technique, as well as encapsulation–vitification and droplet-vitrification have been developed for a very broad range of plant species and for various types of materials like somatic embryos and cell suspensions. However, the encapsulation–vitrification technique combines the advantages of encapsulation-dehydration (ease of manipulation of encapsulated explants) and of vitrification (rapidity of implementation) (Matsumoto et al., 1995). In the droplet-vitrification technique excised explants are loaded, treated with the vitrification solution and frozen in individual microdroplets of vitrification solution placed on aluminium foils, which have been immersed rapidly in liquid nitrogen (Sakai and Engelmann, 2007).

1.4.3.2 Encapsulation-dehydration

Sharma, (2005) has stated that the encapsulation-dehydration technique was first developed by Fabre and Dereuddre (1990). Encapsulation-dehydration methods are based on a successive osmotic and evaporative dehydration of plant cells (Swan et al., 1999). The procedure includes the encapsulation of somatic embryos in calcium alginate beads. Dehydration can be achieved by using an osmoticum (e.g. sucrose) (Lipavska and Vreugdenhil, 1996, Ashmore, 1997) and air drying treatments, followed by direct immersion in LN or controlled cooling to an intermediate temperature before transfer to LN. After storage, the beads are re-warmed at room temperature (Mandal et al., 1999). Additional loss of water has also been obtained by evaporation and subsequent increasing of sucrose concentration in the beads (Dereuddre et al., 1991a).

1.4.3.3 Pre-growth and pre-growth desiccation

In the pre-growth technique, the samples are cultured in the presence of cryoprotectants, and then frozen in LN (Blackesley et al., 1996, Panis et al., 1996). In

plant tissues, the use of preculture treatments can improve freezing tolerance (Vicient and Martínez, 1998). Two kinds of cryoprotectant can be found, penetrating and non-penetrating, based on their ability to cross cell membranes (Perez, 2000). Penetrating substances such as DMSO, glycerol and some amino acids like proline while non-penetrating substances include sugars, sugar alcohols and high molecular weight like PEG (Polyethylene glycol) (Kaviani, 2011). The pregrowth-desiccation system has been referred to as the preculture of the explants on medium to promote desiccation tolerance in order that it can be desiccated and cryopreserved with minimum cryoinjury (Fig. 3) (Sharma, 2005). Desiccation causes stress in the explants; therefore, a preculture process becomes necessary for increasing the cell's resistance to this stress (Melo et al., 2011). The prevention of freezing injury and maintenance of post-thaw viability can be achieved by removing some or most of the water (Gonzalez-Arnao et al., 2008). Studies have suggested that pre-culture of the explants on high sucrose can achieve good results (Blackesley et al., 1996, Dumet et al., 1993a), ABA or proline (Nitzsche, 1980) as well as cold acclimation (Sugawara and Steponkus, 1990).

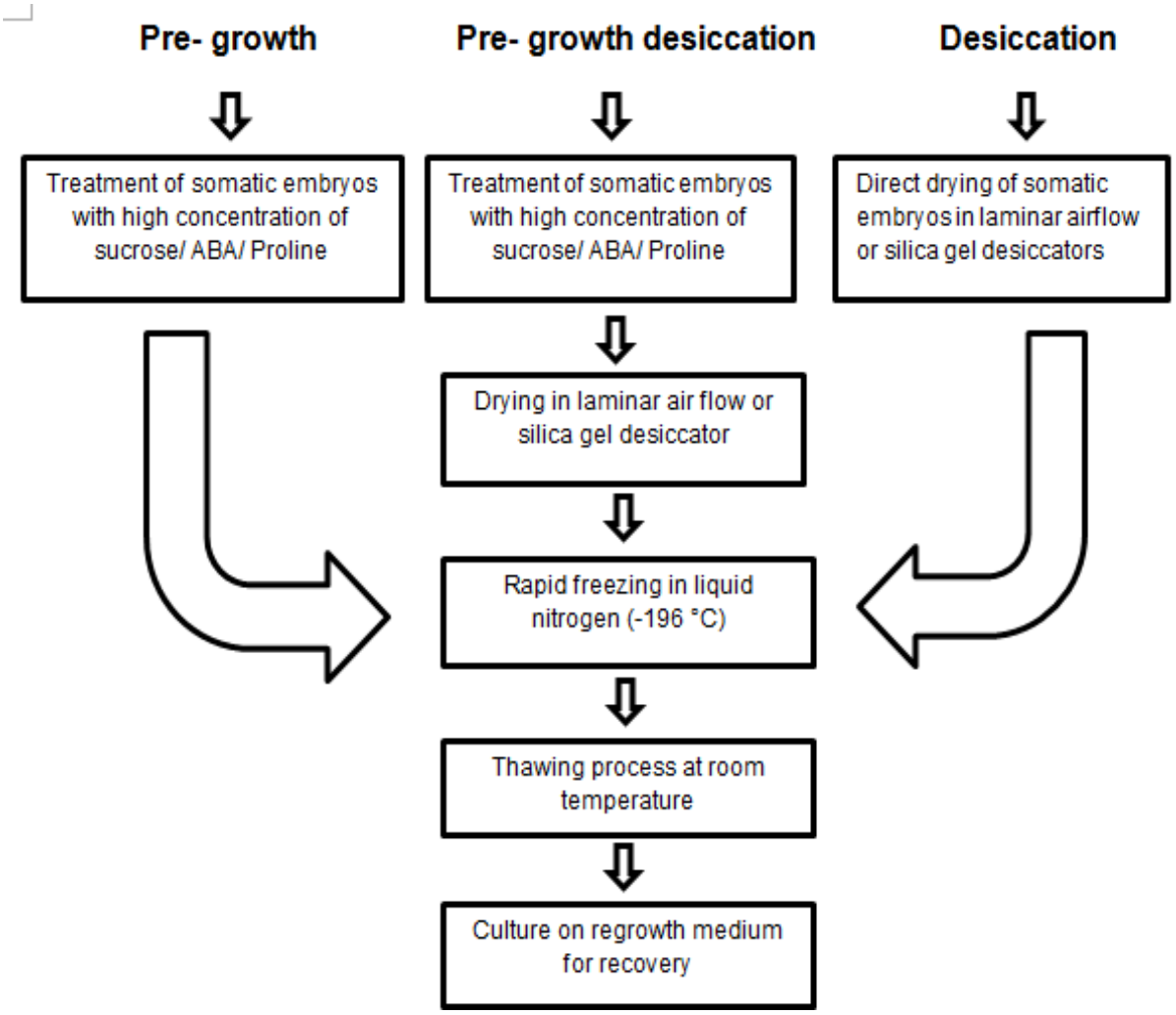


Figure 3. Diagrammatic representation of the process involved in cryopreservation of somatic embryos using pre-growth, pre-growth desiccation, desiccation techniques (modified from Sharma, 2005).

1.4.3.4 Desiccation

Desiccation can be considered as the simplest technique, since expensive freezing equipment, larger storage space and cryoprotector solutions are not required (Popova et al., 2010). Desiccation procedures require only dehydration of the plant material before rapid freezing by direct immersion in LN. Partial desiccation can reduce intracellular water content and reduce ice formation leading to increased freezing tolerance (Vicient and Martínez, 1998). Usually, desiccation of somatic embryos is achieved by placing the embryos in the air current of laminar air flow cabinet for a period of time (Engelmann, 2000). Optimal survival rates can be

obtained when the water content of the embryos is around 10-20% (Dixit, 2001). The induction of desiccation tolerance in somatic embryos can be achieved by culturing embryos in a medium containing a high concentration of sucrose and ABA (Tetteroo et al., 1994, Brown et al., 1993, Lai and Mckersie, 1993, Wang et al., 2003). This method is mainly applied to most common agricultural and horticultural species, zygotic embryos, embryonic axes, orthodox seeds, and pollen grains (Uragami et al., 1990, Engelmann, 2004).

The best technique that can be employed for cryopreservation has been found to be species specific. If the species is cold hardy, such as plants from temperate or subtropical regions, pre growth and/ or pregrowth desiccation can be applied. In the case when the species is sensitive to low temperatures, like the plants growing in tropical regions, encapsulation-dehydration or vitrification can be followed (Sharma, 2005).

1.4.4 Cryopreservation of embryogenic callus tissues

Cryopreservation techniques allow for the conservation of organs and tissues that are produced from *in vitro* culture (e.g., embryogenic callus, somatic embryos and shoot tips) in liquid nitrogen (Lambardi et al., 2008). The main goal of cryopreserving suspension cells and calluses tissues is the conservation of specific features that could be lost during *in vitro* conditions. The classical slow-cooling method (0.5°C min^{-1} up to -40°C) is often used for cryopreservation of these tissues (Panis and Lambardi, 2005). It is also called controlled freezing, slow freezing or the two step freezing method and based on chemical cryoprotection and slow cooling, followed by rapid immersion in liquid nitrogen. In this technique decreasing temperature at a relatively slow rate, ice crystals are formed in the extracellular solution and water is the removed from the intracellular cytosol, leading to cellular dehydration and

therefore avoids intracellular ice formation (Meryman and Williams, 1985). Embryonic calli were successfully cryopreserved with this method for sugarcane (Martinez-Montero et al., 1998, Martinez-Montero et al., 2002). Also, the two main types of new cryopreservation techniques, which are termed vitrification and encapsulation-dehydration (Gonzalez-Benito et al., 2004) have been used to preserve embryogenic callus. The vitrification method has been used successfully to cryopreserve embryogenic cultures of Maize (Xiaomei et al., 2001), *Quercus robur* (Martinez et al., 2003), *Quercus suber* (Valladares et al., 2004), horse chestnut (*Aesculus hippocastanum* L.) (Lambardi et al., 2005), *Anemarrhena asphodeloides* Bunge (Sen-Rong and Ming-Hua), castor aralia (*Kalopanas septemlobus*) (Shin et al., 2012), wild crocus species (*Crocus hyemalis* and *Crocus moabiticus*) (Baghdadi et al., 2011), *Dioscorea bulbifera* L. (Hong et al., 2009) also it was reported that *Dioscorea bulbifera* L. can be cryopreserved by encapsulation vetrification (Ming-Hua and Sen-Rong, 2010). The encapsulation-dehydration technique is widely used because it is applicable to many species (Shibli, 2000). It was applied to preserve embryogenic callus of Date Palm (*Phoenix dactylifera*) (Subaih et al., 2007), wild crocous (*C. Hyemalis* and *C. moabiticus*) (Shibli et al., 2009) and sweet potato (*Ipomoea batatas*) (Blakesley et al., 1995). Moreover, cryopreservation of sugarcane callus was substantialy improved using pregrowth on liquid medium containing 0.33 M sorbitol and avoiding post thaw removal of cryoprotectants (Gnanapragasam and Vasil, 1992). The dehydration technique was also reported to preserve embryogenic callus of hybrid tenera oil palm after preconditioning of callus tissue on MS medium supplemented with 0.25 M sucrose for 7 days (Khawniam and Te-chato, 2012).

1.5 Morphological characteristics of cauliflower somatic and zygotic plantlets

1.5.1 Morphology of cauliflower plant

Cauliflower is an annual plant that reproduces by seed (Kashyap, 2013). There are five stages of development between vegetative growth and flowering which have been recognized (Margara and David, 1978) cited in (Anthony et al., 1996). 1) Vegetative stage. 2) The initiation of inflorescence resulting in the formation of secondary meristems in axils of bracts. 3) The development of curd by the multiplication of meristems. 4) The maturity of curd with no flower initials. 5) Floral differentiation and elongation of some of the inflorescence branches. The stages 2-5 are collectively known as the generative stage. Generally, the curd can be considered an early arrested stage of indeterminate inflorescence development since its formation precedes floral initiation (Paddock and Alexander, 1952, Bayly and Craig, 1962, Bowman et al., 1993). Also, the period from transplanting to harvest can be divided into three phases (Wurr et al., 1981) a juvenile phase, a curd induction phase and a curd growth phase (Olesena and Grevsen, 2000). A juvenile phase can be determined by the number of initiated leaves (Booij, 1990a). In the curd induction stage, the apex is induced to change from vegetative to floral prior to producing a curd. In winter varieties, the curd induction requires an exposure to low temperatures which is called vernalization. The duration of this stage is essentially dependent on the genotype (Kalloo and Bergh, 1993). In the curd growth stage, the vegetative apex becomes generative and grows into the curd. The curd is a prefloral formation which can share some attributes of the reproductive and vegetative apices. In case of not harvested, it is able to grow into flower buds. However, it can lengthen up to 50 cm in suitable environmental conditions (Sadik, 1962, Wiebe, 1975).

Brassica oleracea L. is highly polymorphic including varieties which exhibit a headed phenotype (a large preinflorescence): the curd of cauliflower and `romanesco' (var. *botrytis*) as well as the spear of broccoli (var. *italica*). The highly iterative patterns of activity at the primary meristems result in a headed phenotype for these varieties. A relatively long preinflorescence stage in cauliflower and `romanesco' lead to appearance curd surface being composed largely of branch primordia, whereas the short this stage in broccoli and the spear surface which consists of flower buds (Kieffer et al., 1998) (Fig.4). The cauliflower plant has a small, thick stem which bears whorls of leaves and branched tap root system. The main growing point develops in a shortened shoot system whose apices make up the convex surface of the curd. Generally, the color of the curd is white; this color varies with variety and environment and it might be white, cream-white, green, yellow or red (Board, 2004). The common terms that are used to describe the edible part of cauliflower are head, curd, inflorescence and flower cluster and amongst these curd is most frequently used (Sadik, 1962). The curd can be defined as composed structurally of a number of shortened shoots on which a tremendous amount of naked apical meristems are found (Xiao-Fang et al., 2000). The curd consists of a large 'preinflorescence' with a complex morphology characterized by a high degree of ramification, an accumulation of meristematic domes and a little intermodal extension (Kieffer et al., 1996). After a vernalization period, internodes elongate and floral development ensues (Carr and Irish, 1997). The accumulation of millions of meristems on its surface can occur during curd development (Kieffer and Fuller, 2013). At harvest time, the surfaces of a cauliflower head (the curd) can be distinguished as a dome of tissue consist of a mass of proliferating floral meristems. Only ~10% of these meristems will later

develop into floral primordia and form normal flowers with the rest aborting (Sadik 1962).

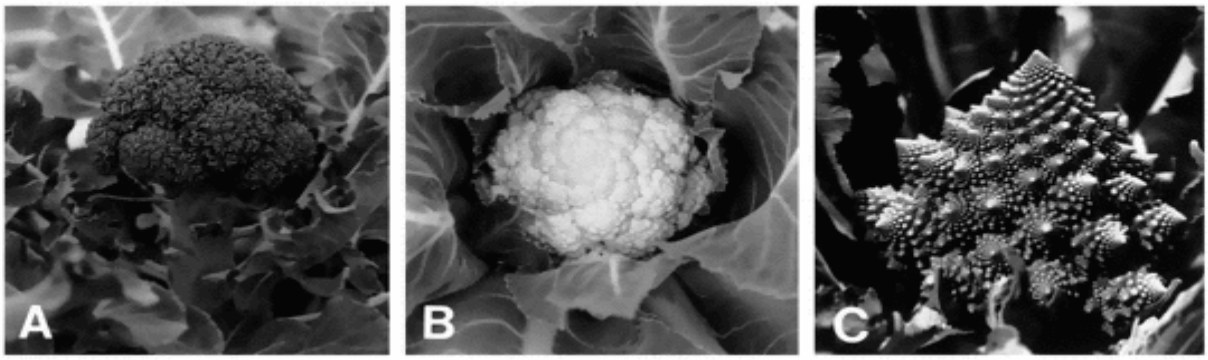


Figure 4. *Brassica oleracea* headed phenotype. A Broccoli spear. B Classical white semi-spherical cauliflower curd. C Green pyramidal 'romanesco' curd. Cited in (Kieffer et al., 1998).

1.5.2 Morphological characteristics through harvesting time

Cauliflower can be harvested when the curds are compact, maintain the original color and attain the appropriate size. White and compact curds which are medium-sized are mostly preferred in the markets. When harvesting is delayed, non-marketable, loose and discolored curds are obtained. To protect the curds from attaining a yellow color after direct exposure to the sun, the leaf is placed on the curd or tying of the tips of leaves immediately after curd development is sometimes practiced. This method is called blanching. In early-mid season varieties having spreading and open plant type this method is quite common. While, most of the later types commonly have self-blanched habit (Board, 2004).

1.6 Aim and objectives of the study

This study aims to:

Improve the *in vitro* regeneration system in cauliflower through improving protocols for production, encapsulation and cryopreservation of somatic embryos.

The objectives are:-

1) Determine suitable concentrations of plant growth regulators to be used to induce embryogenic callus and somatic embryogenesis especially auxins and cytokinins and the best type of medium (semi solid or liquid) which is suitable for embryogenic callus proliferation.

2) Determine the best explants for callus induction and for somatic embryogenesis. Also the best date for subsequent callus culture.

3) Determine the best concentration of sucrose to induce somatic embryogenesis as well as to evaluate the best culture system (temporary immersion and continuous immersion in liquid medium) and develop the most efficient for somatic embryo production.

4) Study the ability of primary somatic embryos to produce secondary somatic embryos.

5) Optimize the best matrix for encapsulation of somatic embryos to produce artificial seed.

6) Evaluate the response of somatic embryos and embryogenic callus to different technique and durations of exposure to liquid nitrogen and investigate their ability for cryopreservation.

7) Investigate the phenotypic variation between the plants which produced from somatic embryos and plants that produced from seed through morphological studies on plants in the field.

Chapter two

Callus induction and proliferation

2.1 Introduction

2.1.1 Effect of growth regulators on callus induction and proliferation

In vitro small embryoids resembling the zygotic embryos which can be produced from the embryogenic somatic plant cells of callus tissue or of suspension cultures are known as somatic embryogenesis (Kumar, 1995). Plants can normally be propagated from unorganized callus tissues derived from various explants induced by exogenous growth regulators (Flick et al., 1983). Callus production has been shown to be affected by the type of explants, concentration, combination and type of growth regulators in the medium (Comlekcioglu et al., 2009). For successful callus yield and plant propagation, the concentration of plant growth regulators (auxin and cytokinin) are critical but precise types and concentrations needed can be specific to genotype, explant type (Ahmad and Spoor, 1999) and the needs of the project (Dunwell, 1981). Thus they require optimization. It is necessary to determine the appropriate plant growth regulator balance for both induction of callus and for subsequent callus growth (Ahmed and Spoor, 1999). Many workers have already optimized growth regulators for callus production in different species (Dietert et al., 1982, Murata and Orton, 1987, Das, 1991, Yang et al., 1991) and it has been reported that many types of commercially available auxins and cytokinins are used successfully for tissue culture of Brassica species (Ahmad, 1996).

2.1.2 Effect of explants type on callus induction and proliferation

Different explants including cotyledon, hypocotyls and root from Brassica seedlings can be used to induce callus production (Fuller and Fuller, 1995). Callogenesis can be induced in cauliflower from hypocotyls and cotyledon (Raut, 2003, Ying Qin, 2006) but there is little evidence in the literature of embryogenic callus for cauliflower. Regeneration of embryogenic calli can be applied as one of the most

powerful aspects of somatic embryogenesis for applications such as mass propagation and gene transfer (Karami, 2007).

2.1.3 Culture systems

Liquid culture technology can save on laboratory operational cost including, time, labour and chemicals. Also the quality of plant products in liquid medium is often improved compared to semi solid medium (Gupta et al., 2003). Especially agitated liquid media as this type of culture prevents explants from drowning in the liquid by the continuous rocking motion of th platform (Metwali and Al-Maghrabi, 2012). Solid medium tends to promote the growth of plants whereas; the liquid medium promotes the regeneration of adventitious plantlets. (Te-chato and Muangkaewngam, 1992). The main target of the present experiment was to establish an efficient system for induction and proliferation for callus cultures of cauliflower.

2.2 General materials and methods

2.2.1 Seed germination and explants preparation for callus induction

Seeds of cauliflower cv White Cloud, which is commonly cultivated in large regions of Iraq, were used to produce seedlings. Seeds were surface sterilized in 50% by volume commercial sodium hypochlorite solution (NaOCl 0.06%) for 10 min with 2 drops of Tween 80 as a surfactant and wetting agent, followed by three rinses with sterile distilled water and then 90% ethanol for 1 min followed by three rinses with sterilized distilled water and then germinated on hormone free MS medium (Murashige and Skoog, 1962) (basal salt medium 4.4 g L^{-1}, 30 g L^{-1} sucrose, 7 g L^{-1} technical agar). The seeds were incubated under 16 photoperiod at 80 µmol m^{-2} s^{-1} at 22.5 ^{o}C. After 7–10 days seedlings were removed and cotyledon and hypocotyl segments (5 mm in length) and root segments (5-10 mm) were excised using fine sterile forceps and a scalpel and used as explants and placed on callus induction

medium (CIM) which consisted of MS (Murashige and Skoog, 1962) basal medium supplemented with 0.15, 0.5, 1.0, 1.5, 2.0 mg L^{-1} 2, 4-dichlorophenoxy acetic acid (2,4-D) and 0.1, 0.5, 1.0, 1.5, 2.0 mg L^{-1} Furfurylaminopurine (Kinetin), 30g of sucrose, 7g of technical agar as well as a control (without hormones).

2.2.2 Subsequent culture on semi solid media

The primary callus derived from hypocotyls and root explants after 28 days of culture on CIM was excised carefully to ensure no original explant material was included. This was then cut into small 2 mm diameter pieces manually and sub-cultured on the same semi solid CIM medium. Subsequently the best four CIM media were chosen and used as follows:

1) 0.5 mg L^{-1} 2, 4-D + 0.5 mg L^{-1} Kinetin

2) 0.5 mg L^{-1} 2, 4-D + 1 m gL^{-1} Kinetin

3) 0.5 mg L^{-1} 2, 4-D + 2 mg L^{-1} Kinetin

4) 0.15 mg L^{-1} 2, 4-D + 0.1 mg L^{-1} Kinetin

Cultures were distributed randomly inside incubator shelves. Callus diameter and its color were recorded 7, 14, 21 and 28 days after sub-culturing. A ruler was used to measure the callus diameter.

2.2.3 Subsequent culture using a continuous immersion in agitated liquid medium technique (CI ALMT).

Explants were produced using this technique described as follows:

A commercial blender (Multi-mixer model no. 50376) (Fig. 5 A) disinfectant in 50% commercial bleach for 30 mins followed by immersing in 70% ethanol for 10 min and rinsed three times in distilled water.

Hypocotyl and root derived callus tissue was excised and then disrupted into small pieces using a blender. Blending step was made in MS medium.

The explants produced were classified into various size classifications using commercial sieves (Endecotts Ltd., London) (Fig. 5 B).

Small callus pieces 1-2 mm were obtained after 75 seconds blending. Although this size was produced significantly ($P < 0.001$) from all blending durations used (15, 30, 45, 60, 75, 90 sec) but we used 75 seconds as the highest amount of callus tissue in terms of fresh weight and volume can be achieved using is period as it will be mentioned in chapter three section (3.2. 3).

After sieving, constant volumes of callus explants were taken using a precise volumetric measure (74 uL), and were placed in pots contained 30 mL of liquid CIM Media per pot (the best four CIM media mentioned above in section 2.2.2).

Callus cultures were agitated at 150 rpm using a rotary shaker (Fig. 5 C) in the laboratory and supplemented with 16h light (spectral photon fluency 40 µmol m^{-2} s^{-2}) supplied by cool white fluorescent tubes. Callus characteristics during subsequent culture were recorded after 7, 14, 21 and 28 days.

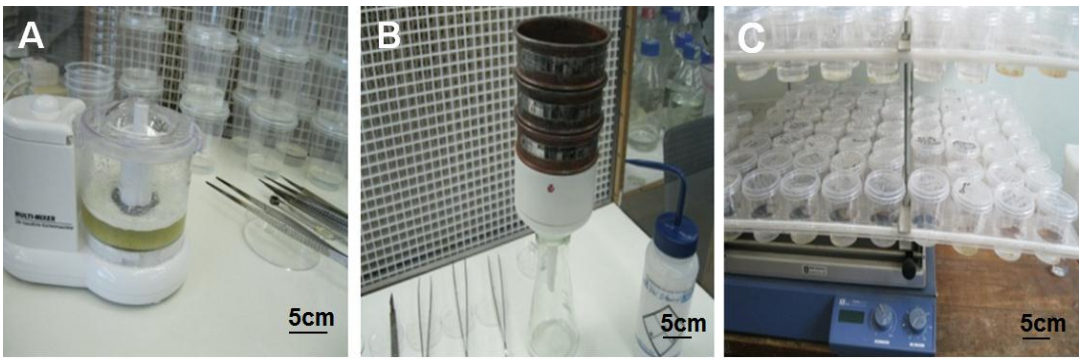


Figure 5. A) commercial blender (multi-mixer model no. 50376). B) Sieves (Endecotts Ltd., London). C) Rotary shaker.

2.2.4 Statistical analysis

All experiments were set up in a randomized complete block design (RCBD) with incubator shelves used as blocks and the experiments of subsequent culture on semi solid and liquid medium were set up in a factorial design. All data were subjected to analysis of variance (ANOVA) using Minitab software (version 16) and comparison of means were made using the least significant difference test (LSD) at 5 % probability. All graphs were plotted using Microsoft Excel 2010. All data were tested for normality distribution using Minitab Basic statistics which showed the data were normally distributed and did not require transformation. Results in graphs are presented as means ± standard error (S.E.).

2.3 Experiments

2.3.1 The effect of exogenous growth regulators and type of explants on callus production

2.3.1.1 Objective

To study the effects of explant type and different concentrations of auxins and cytokinin on the callus induction of cotyledons, hypocotyls and root segments.

2.3.1.2 Material and methods

Cotyledon, hypocotyls and root segments of 7 days old seedlings were placed on callus induction medium (CIM). The pH was adjusted to 5.8 before autoclaving for 15 min at 121 ^{o}C and 1.07 kg cm^{-2}, 20 m L of medium was poured into 9 cm sterile petri dishes under aseptic conditions in a laminar flow cabinet. Explants were cultured on petri dishes containing 5 explants with 5 replicates, individual petri dishes were sealed with parafilm in order to reduce contamination and to maintain medium moisture content during culture. After 28 days the callus diameter and morphogenetic characters were recorded.

2.3.1.3 Results

Embryogenic callus tissue (ECT) production from all types of explants used was achieved in this study with several hormone combinations. The results showed that the use of 0.15 mg L^{-1} 2,4-D + 0.1 mg L^{-1} Kinetin as exogenous hormone combinations in CIM was the optimal combination producing the highest mean callus diameter (Fig 6). It was observed that there were highly significant differences between this treatment and all other treatments. The callus in the optimum treatment was also friable and bright green in colour indicative of a good quality. Callus initiation and proliferation on medium with 0.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kinetin was not significantly more than that on the media supplemented with 2/1.5, 2/2, 2/1, 0.5/1.5, 0.5/2, 1/1 mg L^{-1} 2,4-D and Kinetin, but significantly more than that on all other media types. Good callus appearance was also found on this medium, being bright green and friable but it showed significant variation in values of callus diameter. In contrast watery callus was observed on media with 2/1.5 and 2/2 mg L^{-1} of 2, 4-D and Kinetin and it was found that the use of 2, 4-D at 0.5 mg L^{-1} with Kinetin at 1/1.5/2 mg L^{-1} had a positive effect on callus induction in terms of callus diameter, callus color and texture with the callus being friable and bright green. No callus growth was observed on the medium without growth regulators or on media devoid of auxin. The weakest response for callus induction was on medium with 1 mg L^{-1} 2, 4-D and devoid of Kinetin.

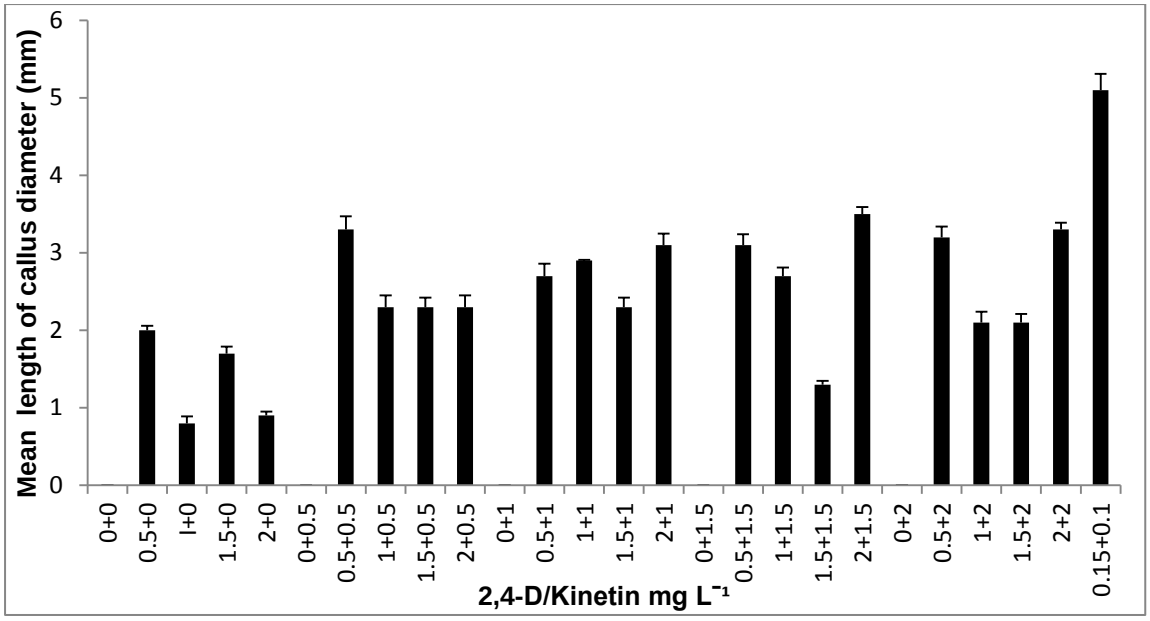


Figure 6. Effect of different levels of 2, 4-D and Kinetin that added to the CIM on mean callus diameter that produced from various cauliflower's explant types (Cotyledon , Hypocotyls and Root) after 28 days of incubation (LSD= 0.6).

The explants were swollen after 7 days of culture and the callus appeared within 14 days incubation on CIM, however, after 28 days the mean callus diameter varied greatly among the explants (Fig. 7). A bright green friable putatively embryogenic callus proliferated on the cut edges of the hypocotyl explants which grew after initiation (Fig. 8). Hypocotyl explants showed better capacity for callus induction in terms of callus diameter, color and texture. Also significant variation in values of callus diameter was noted from root explants which produced a more yellow friable callus. Cotyledon explants were the least responsive for callus production. Significant interaction differences were found in the terms of calli diameter between exogenous hormone concentrations and the type of explants. The highest callus diameter achieved was from the use of hypocotyls on medium with 0.15 mg L^{-1} of 2, 4-D + 0.1 mg L^{-1} of Kinetin (Fig. 9).

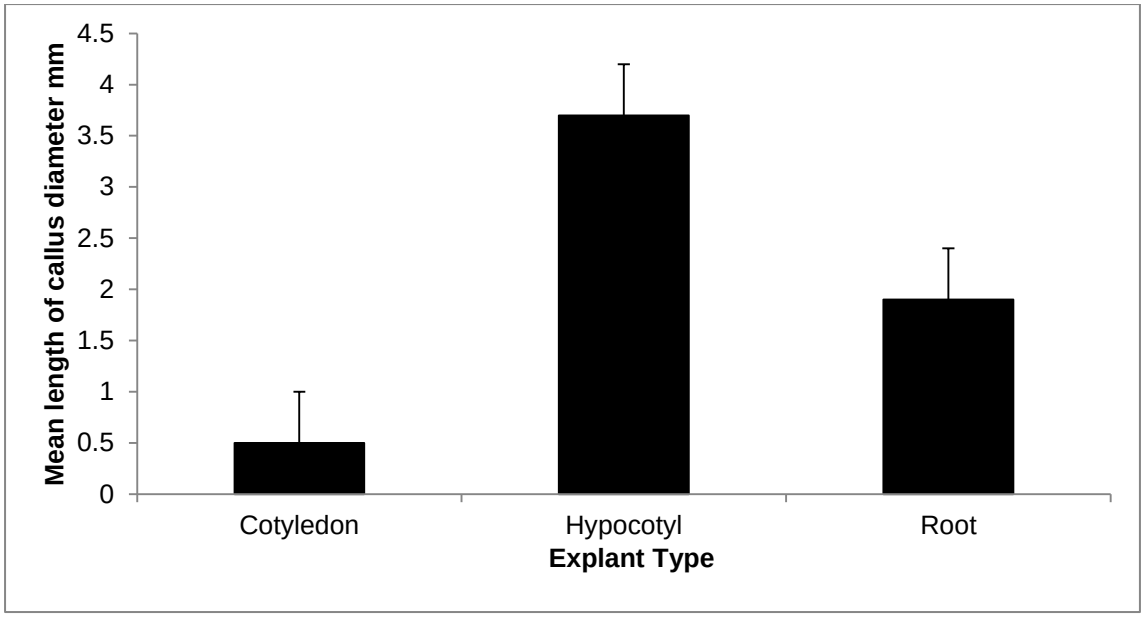


Figure 7. Overall effect of various explants (Cotyledon, Hypocotyl and Root) on mean callus diameter after 28 days of culture on CIM. (LSD= 0.2).

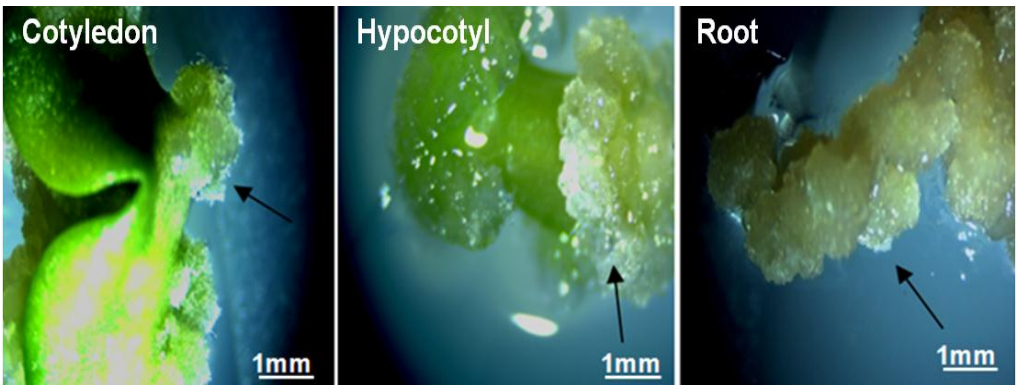


Figure 8. Photographs of calli initiated on explants (Cotyledon, Hypocotyls and Root) after 28 days from culture on CIM.

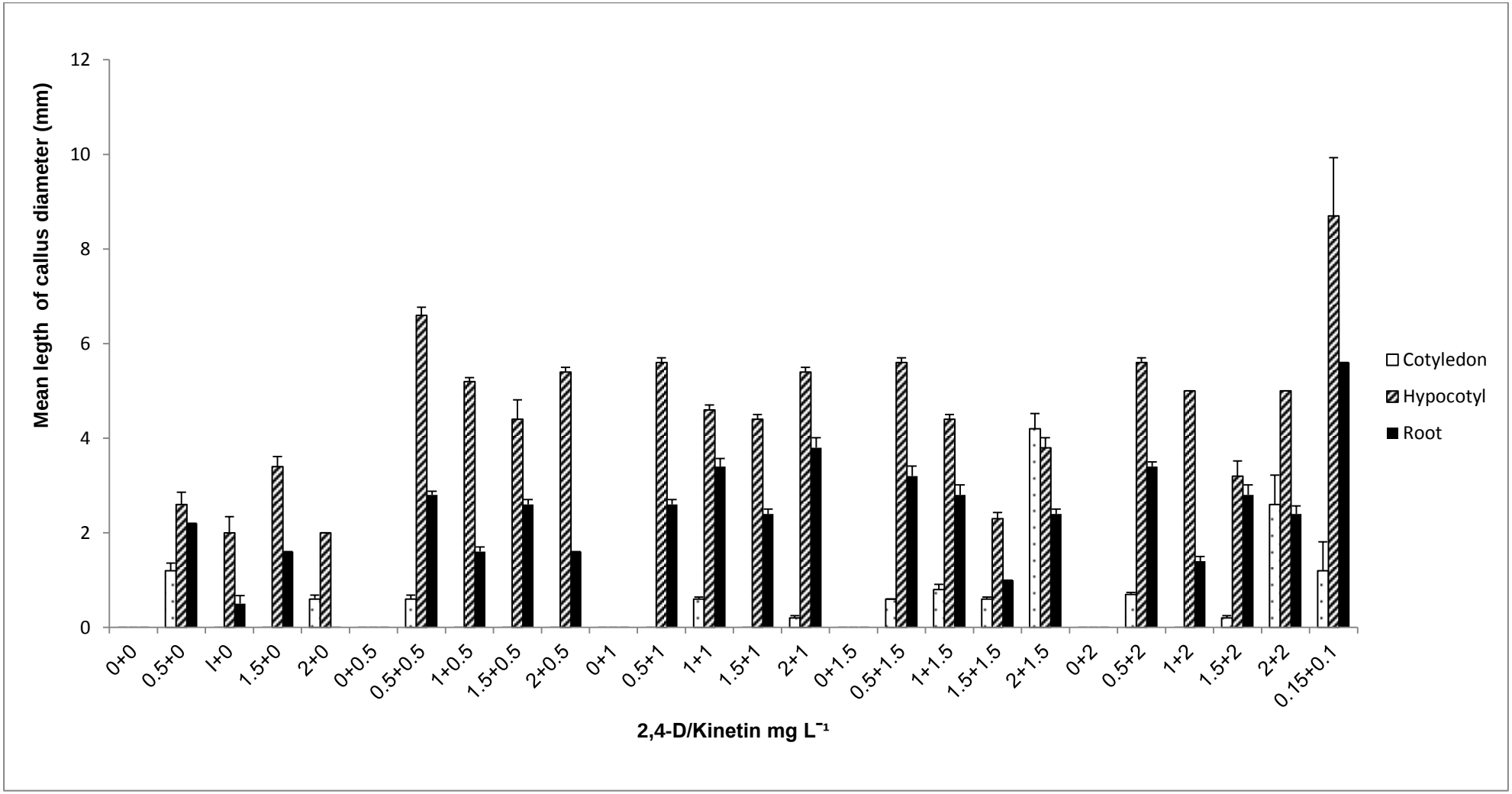


Figure 9. The effect of exogenous hormone combinations and explants type on values length of callus diameter (LSD = 1.1).

2.3.2 The growth ability during subsequent callus culture of semi solid media

2.3.2.1 Objective

To investigate the capacity of callus tissue explants for proliferation during subsequent culture on semi solid medium.

2.3.2.2 Materials and methods

Callus tissues were excised from explants and dissected into small pieces (2 mm) for sub-culture. Five pieces of callus in petri dishes containing the best four semi-solid CIM media that were identified in section 2.2.2.

2.3.2.3 Results

The results revealed that the frequency of callus growth during subsequent culture was higher in callus obtained from hypocotyls than from that obtained from root explants ($P < 0.001$) (Fig. 10). Furthermore it was bright green and friable on ex-hypocotyl explants and, yellow and friable on ex-root explants. Maximum callus growth was initiaited on the periphery of the primary callus after 28 days of culture. Also, it was observed that after 21 days of subculture, most callus tissue turned brown. At the same time, a new callus tissue continuous to form on the old pieces and therefore, this period (21 days) was considered the best for subsequent embryogenic callus culture (Fig. 11). The media with 0.15 mg L^{-1} 2,4-D + 0 .1mg L^{-1} Kinetin as well as 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kinetin were significantly better than other media (Fig.12). A significant interaction was found between exogenous hormone combinations, type of explants and days which were used during sub-culture ($P < 0.03$).

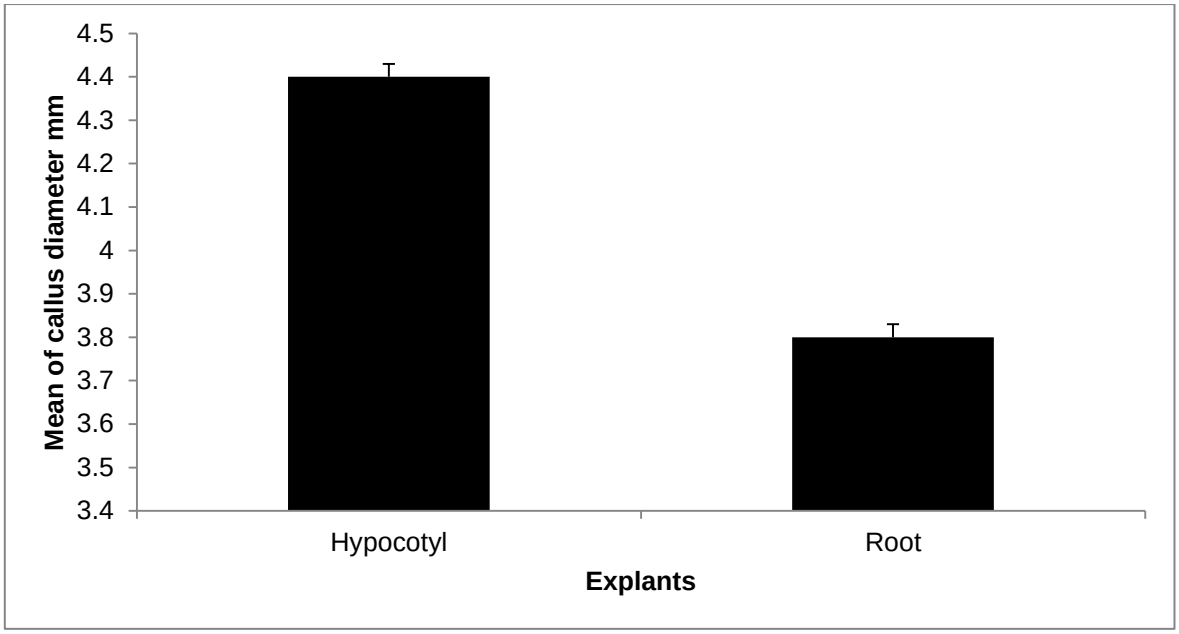


Figure 10. The effect of explant origin type on callus growth during subsequent culture on semi solid media (LSD = 0.2).

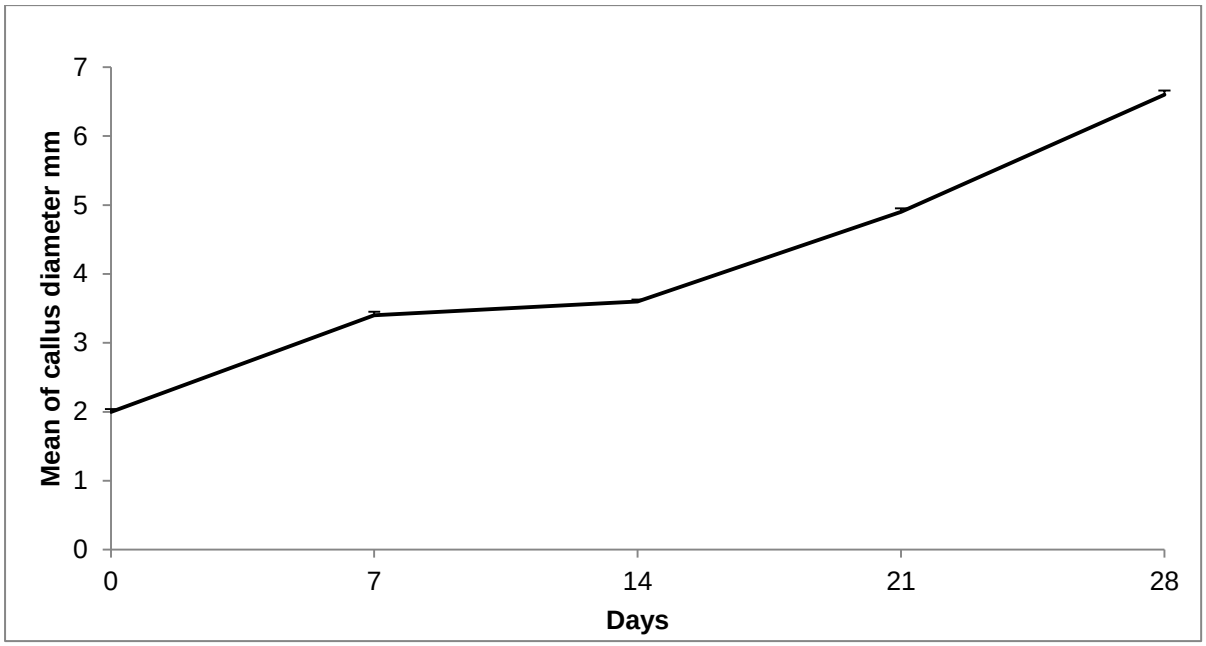


Figure 11. Callus growth during subsequent culture on semi solid media (LSD = 0. 4).

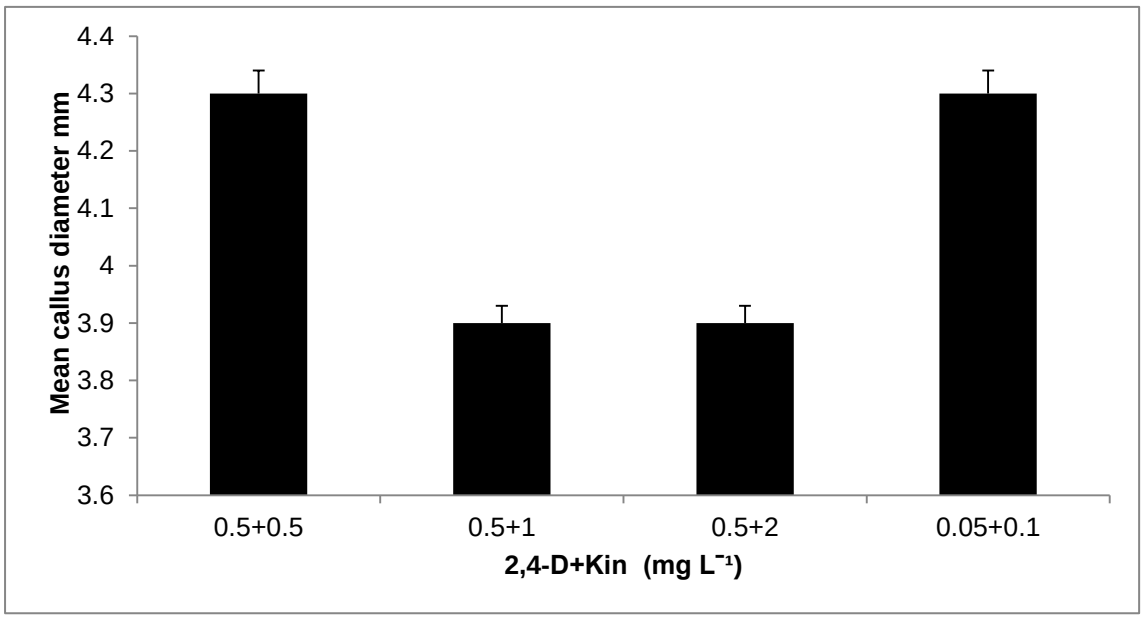


Figure 12. The effect of exogenous hormone combinations of 2, 4-D and Kinetin on callus diameter during subsequent culture on semi solid media (LSD = 0. 4).

2.3.3. The effect of sterilants (sodium hypochlorite solution and ethanol) that used to sterilize a blender on levels of contamination occoured.

2.3.3.1 Objective

To investigate the efficiency of disinfectants for sterilization the equipments of plastic blender which used to disrupt the callus tissue in the current study.

2.3.3. 2. Materials and methods

Different treatments were used for sterilization of a plastic blender that used in disruption of callus tissues and described as follows:

1) Without sterilization (control).

2) Soaked in 70% ethanol for 30 min.

3) Soaked in 50% commercial bleach (sodium hypochlorite solution) (NaOCL) (v/v) for 30 min.

4) Dipping in 50% commercial bleach for 30 mins followed by immersing

in 70% ethanol for 10 min.

These were followed by rinsing three times with sterilized distilled water. After that, Callus tissues were disrupted using a blender. Explants (74 uL) were cultivated in each plastic pot (9.0 cm inner diameter at the top and 5.0 cm at the bottom), Thirty plastic pots each containing 30 mL of liquid CIM medium were used for every treatment with six replications.Cultures were kept on rotary shaker, levels of contamination was recorded after a few days of culture.

2.3.3.3 Results

The results showed that the use of disinfectant agents gave significant variation in terms of reducing the level of contamination. It was found that the lowest level of contamination (6%) was achieved by disinfecting the plastic blender with 50% commercial bleach for 30 min and followed by immersing in 70% ethanol for 10 min, this treatment differed significantly from all other treatments used. Also, It was observed that immersing in 70% ethanol for 30 min did not differ from immersing in 50% bleach for 30 min where the percentage of contamination was 13% and 10% respectively, while it differed significantly from control ($P < 0.001$). The highest level of contamination (90%) was achieved when the blender was without sterilization and showed that the contamination could reduce the level to 6% by sterilization (Fig.13).

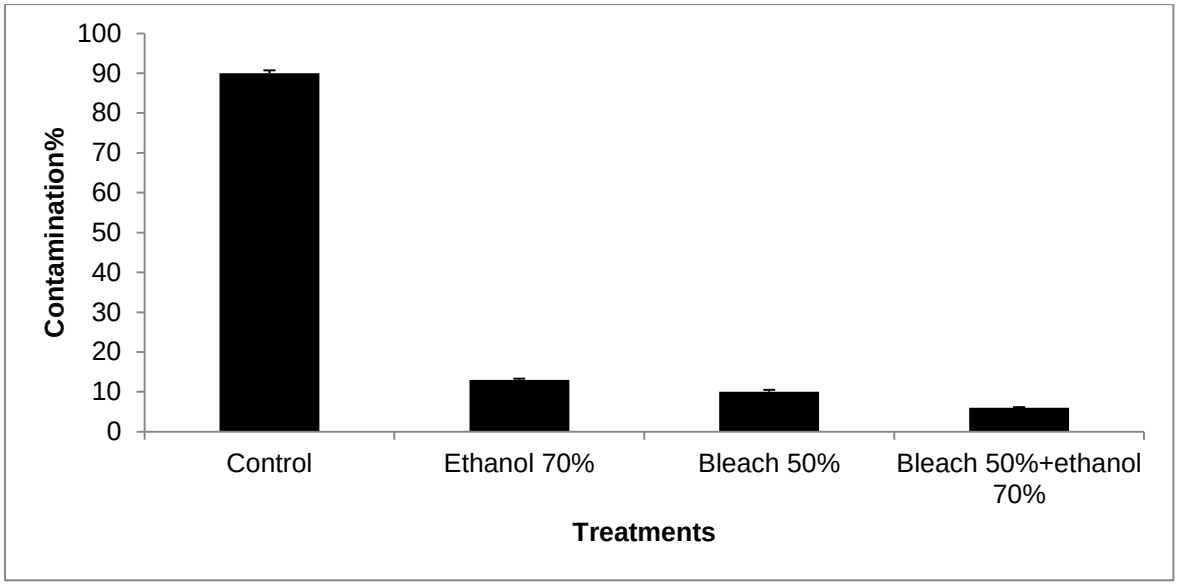


Figure 13. The effect of sterilants that used for sterilization of a plastic blender on level of culture contamination (LSD = 3.6).

2.3.4 The growth ability during subsequent callus culture in agitated liquid media using a continuous immersion in agitated liquid medium technique (CI ALMT).

2.3.4.1 Objective

To study the proliferation capability of embryogenic callus tissue in in agitated liquid medium.

2.3.4.2. Materials and methods

Hypocotyls and root derived callus tissue pieces (2 mm) were produced using CIALMT. Five replicates were conducted for each treatment, and each replicate was represented by a plastic pot containing 30 mL of CIM media (as described in section 2.2.3) and 74 µL of explants were cultivated in each pot, cultures placed at random on a rotary shaker and cultured. Callus diameter, color and texture were recorded after 7, 14, 21 and 28 days.

2.3.4.3 Results

It was observed that small aggregates of embryogenic cultures formed when callus tissue was chopped and subcultured in agitated liquid medium (Fig.14). The use of 2,4-D at 0.15 mg L^{-1} and Kinetin at 0.1 mg L^{-1} was the optimum where callus had grown to 3.4 mm in diameter and this was statistically bigger ($P < 0.001$) compared with the other media used (Fig. 15). The greatest embryogenic callus diameter was achieved on hypocotyl-derived callus ($P < 0.001$) and it was characterized as friable bright green in terms of callus color and texture. Root- derived embryogenic callus was friable and yellow (Fig. 16). Also, the results showed that callus cultures turned brown after 21 days (Fig. 17).

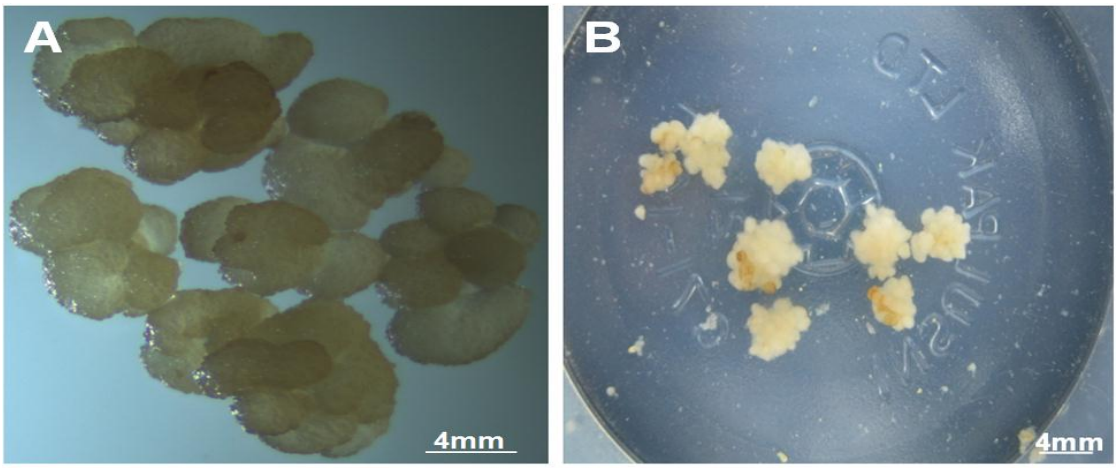


Figure 14. The effect of liquid callus induction medium (CIM) containing (0.15 mg L^{-1} 2, 4-D and 0.1 mg L^{-1} Kinetin on growth during subsequent culture. A) Callus tissue derived from hypocotyl explants. B) Callus tissue derived from root explants.

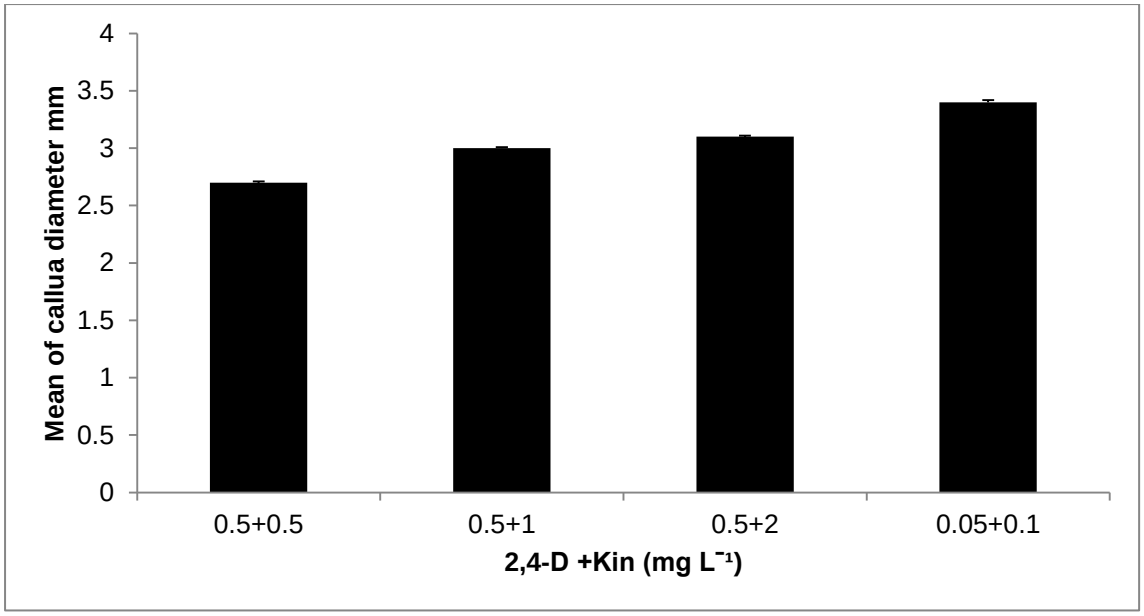


Figure 15. The effect of exogenous hormone combinations of 2, 4-D and Kinetin on callus diameter during subsequent culture in liquid media (LSD = 0.1).

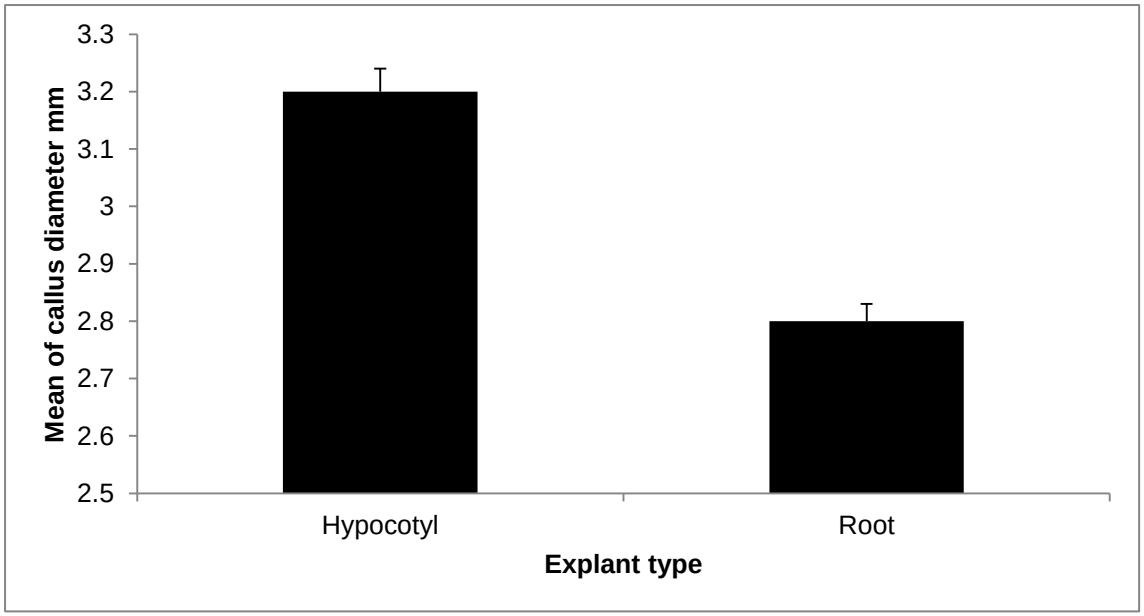


Figure 16. The effect of explant origin type on subsequent callus growth in liquid media (LSD = 0.1).

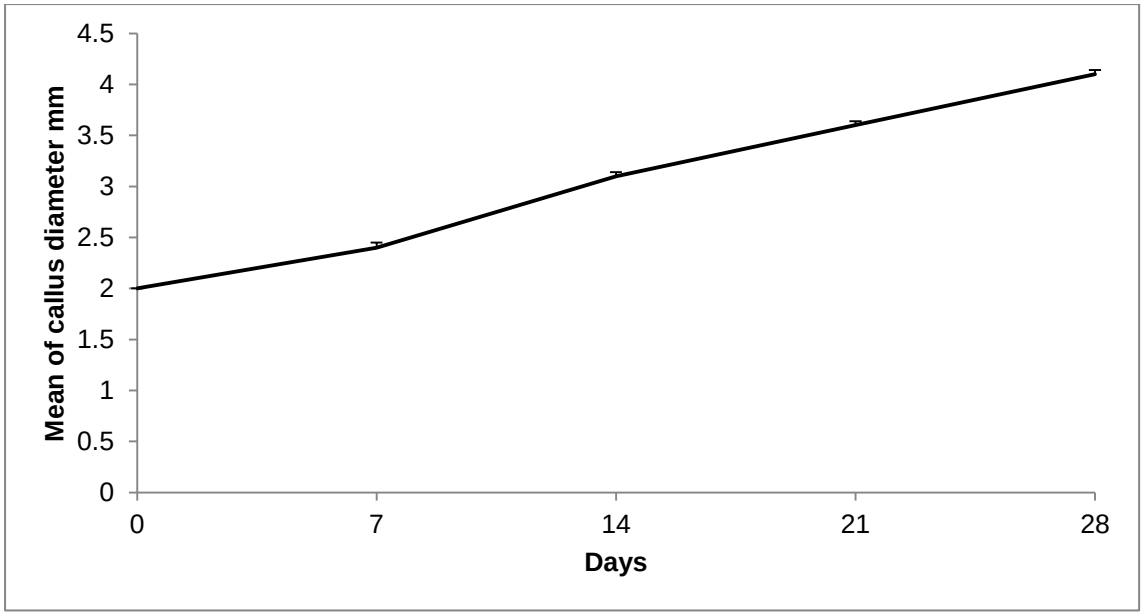


Figure 17. Callus growth during subsequent culture in liquid media (LSD = 0.1).

2.4 Discussion

2.4.1 Plant growth regulator effect

Efficient callus induction and proliferation was achieved under this current study procedure. The putative embryogenic callus tissue was produced most optimally from cauliflower explants on medium containing 2, 4-D and Kinetin as previously described by (Leroy et al., 2000, Raut, 2003). Callus production was affected by the concentration, combination and type of exogenous hormone as well as the type of explants and this is in agreement with (Comlekcioglu et al., 2009). Results also showed that lower concentrations of 2, 4-D and Kinetin used with CIM produced the highest callus diameter. This contrasts with the results reported by Leroy et al (2000) who indicated that callogenesis was induced on hypocotyl explants of cauliflower when the medium was supplemented with a slightly higher concentration of 2,4-D (1 mg L^{-1}) and Kinetin (1 mg L^{-1}). Also, these findings are in contrast with Omidi and Shahpiri (2003) who used higher concentrations of both 2, 4-D and

Kinetin (5 mg L-1 and 0.25 mg L-1 respectively) to induce callus from leaf and internodes explants of potato. The findings reported here indicated that there was a good callus growth when the 2, 4-D and Kinetin were in 1:1 ratio (i.e. 0.5 mg L^{-1} each). These accords with (Ahmad and Spoor, 1999) who found that the optimum concentration for callus production in curly kale (a *Brassica oleracea* subspecies closely related to cauliflower) was on a medium having auxin and cytokininin in balance. Also Mungole et al (2009) found the highest percentage callus response was achieved from leaf explants of *Ipomoea obscura (L.)* in combinations of 2, 4-D (0.8 mg L^{-1}) with Kinetin (0.8 mg L^{-1}). Comlekcioglu et al (2009) as well as Ahmad and Spoor (1999) have reported that there was no callus observed from explants types on hormone free MS and this was confirmed here for cauliflower.

Three kinds of callus morphologies were apparent on CIM, dry bright green friable, dry yellow friable and loose watery callus which gradually turned brown and died and this could have affected subsequent callus induction. These accords with the findings of Stewart et al (1996) as well as Cardoza and Stewart (2004) who mentioned that the main reason for the lower callus induction was hyperhydration which can retard the growth of the tissue. Hyperhydration appears to occur due to either high levels of cytokinin, high temperature or the type of the culture vessel used. These stresses induce more water uptake and cells become over turgid and incapable of division and subsequent proliferation. Ishak et al. (1992) reported that the concentration (w/v) of carbon sources could also play an important role in callus induction and development and when mannitol or sorbitol was added with sucrose callus has been noted to become harder instead of being loose or watery. This could be due to the osmotic potential difference between the explants and the medium. In terms of the current study, watery callus was only observed when high levels of

cytokinin were used but variations in culture vessel, temperature and carbohydrate source were not investigated.

2.4.2 Explants effect

Various explants of cauliflower have been used for initiation of callus including hypocotyls, cotyledon and leaf tissues (Pareek and Chandra, 1978, Leroy et al., 2000, Raut, 2003, Deane et al., 1997, Ying Qin, 2006). Hypocotyl and root explants of Australian cauliflower produced more callus than cotyledon explants (Prem, 1998). Such findings are consistent with the current results. Hypocotyl segments have frequently been reported as the most desirable for plant tissue culture and are the most used for most Brassica species (Cardoza and Stewart, 2004, Ali et al., 2007). The region of the hypocotyl with maximum regenerative ability has also been reported to vary according to the variety of plant and the growth regulators added in the medium (Bigot et al., 1977). Our results are in agreement with many previous studies on cauliflower (Raut, 2003), other Brassica species (Khan et al., 2002) and cotton (Rajasekaran et al., 2004, Zhang, 2000, Zhang et al., 2001) which clearly showed that hypocotyl explants were the most responsive to callus induction and proliferation. This may probably be due to the existence of a number of cells in the hypocotyl region undergoing division which leads to having a greater chance to form callus (Puhan and Rath, 2012).

It was observed that the callus formation was at the cut edges of hypocotyl explants. The addition of auxin and cytokinin to culture medium might reinforce the normally observed polarity of explants and enhance regeneration from unresponsive parts of organs or lead to the disappearance or reversal of polar trends within the auxin accumulation at the basal end of an explants (George et al., 2008). The natural polarity of regenerative events is normally supposed to be due to the natural

movement of endogenous growth regulators through plant tissues, particularly the polar transport of auxin from the shoot apex towards the root tip (Thakur and Ganapathy, 1978). Loss of polarity following the application of growth substances in media has been demonstrated in cauliflower, begonia and rapeseed. Cauliflower petiole explants could produce shoots and roots at their proximal pole when cultivated on media devoid of growth regulators, but this polarity was cancelled if the medium was supplemented with 1.9 mg L^{-1} NAA (α-naphthalene acetic acid), when numerous roots were formed all over the explants. Also shoots appeared all over the explants when 2.3 mg L^{-1} BA was administered instead (cited by (George et al., 2008). Similarly, Akmal et al., (2011) have reported the formation of embryogenic calli on the cut end of both hypocotyls and cotyledon derived-embryogenic calli of mustard was more than other parts of segments used (*Brassica juncea* L.cv. Pusa Jai kisan).

2.4.3 Growth of ECT through subsequent culture

Callus normally requires multiple sub-cultures before embryogenesis can be induced (Zhang et al., 2001) and semi-solid medium is frequently thought to be best for the subsequent development of organs (Chen and Galston, 1967). The development of callus tissue of *Vicia faba* was observed on semi solid medium (Grant and Fuller, 1968). The results presented here confirmed that callus tissue could be induced and proliferate on the same semi solid CIM used as was similarly found with orchid species (Lee and Lee, 2003). Although, the use of semi-solid systems has achieved successful results in terms of proliferation yields, the improvement of productivity and a reduction in the time taken to increase the yields is still a goal in most regenerations systems and agitation utilizing liquid culture medium can be used instead of conventional semi-solid growing techniques. Close contact of the tissue

can be achieved using liquid medium which promotes the uptake of exogenous hormones and nutrients leading to better growth. Continuous shaking of the medium encourages optimal oxygen supply to the tissue (Mehrotra et al., 2007). The ability of callus proliferation has previously been reported for some wild species of Brassicaceae on liquid medium (Toriyama et al., 1987) and has been used with Spanish grape vine cultivars “Albarino” and “Tempranillo”(Gonzalez-Benito et al., 2009), *Heveabra silienies* (Wilson et al., 1976) and Date palm (Sane et al., 2006). The present study indicated that growth of embryogenic callus tissue was more on semi-solid medium. Similar results were obtained by Simonsen and Hildebrant (1971) who showed that callus production from isolated Gladioulus cormel stem tips were more frequent on agar than in liquid media. In contrast with Gupta et al. (2005) who commented that the growth rate on semi-solid media is slow and the uptake of nutrients, water and exogenous hormones has been reduced by gelling agents. On the other hand, it was observed in the current study that chopped callus had a very embryogenic behavior when sub-cultured in liquid medium. This is in agreement with the observations of Kamo et al. (2004) and Sane et al. (2006) on hybrid tea roses and Date palm respectively. However, through subculturing, our results indicated that the frequency of callus formation from hypocotyl-derived callus was higher than root-derived callus and this difference might be due to cellular totipotency of hypocotyl explants (Zelcer et al., 1984, Niedz et al., 1985).

Oluwaseun and Erhinmeyoma (2005) reported that calli of *Parkiabiglobosa* turned friable and more nodular when it was sub-cultured in media containing a combination of 2,4-D and Kinetin. Also Sun et al (2003) indicated that the use of 2,4-D and Kinetin were essential for callus induction and maintenance of cultures and were also loose and green in their morphology which is in accordance with the results

presented here. It was found however that the cultures could become brown and die when the sub-culture interval was longer than 21 days. This finding is consistent with Harry and Thorpe (1991) who mentioned that cell browning could be observed when the subcultures interval exceeded 30 days who speculated that this could be due either to culture exhaustion of a limiting factor or a spatially induced gradient of a limiting factor in the callus itself.

2.4.4 Sterilant agent effects

The homogenization technique was successfully applied to produce a uniform size of explants as has been reported for the proliferation of ferns, bud clusters of potatoes, banana and gladiolus (Ziv et al., 1998) and also for gametophytic and sporophytic tissues of *Aspleniumnidus* (Fernandez et al., 1993). This technique has also been used for the rapid isolation of oilseed rape (*Brassica napus L*) microspores (Polsoni et al., 1988) from flower buds. The mass propagation of cauliflower from fractionated and graded curd has been reported for cauliflower (Kieffer et al., 2001, Rihan et al., 2012, Kieffer and Fuller 2013) where the meristematic layer was disrupted to produce analogous size of explants, to that used here for callus, and an agitated liquid medium was also used to produce shoots. The use of a blender for aseptic homogenization of cultures has also been devised to achieve the rapid production of suspension cultures from callus (Williams et al., 1988, Chen and Galston, 1967). The sterilization of instruments and culture media must be applied to exclude microorganism contamination (Bottino, 1981). Contamination compromises the development of all *in-vitro* techniques, and is identified as constant problem (Enjalric et al., 1988). In plant tissue and cell culture bacteria, yeast and filamentous fungi can be considered as the most common contaminants (Leifert and Cassells, 2001). Some of contamination could emanate from contaminated tools, which have been

not investigated fully or systematically (Odutayo et al., 2007). The use of bleach and ethanol as sterilizing agents are commonly applied in the laboratory and may be preferable to autoclaving instruments and equipment such as blenders which are not designed to withstand repeated autoclaving. Oxidising agents including bleach can be used to attack essential cell components including protein, lipid and DNA and kill microorganisms (Jang et al., 2008). Other disinfecting agents such as ethanol can be applied as a dehydrating material of protein leading to deactivation of the enzymes for growth of bacteria in particular (Cronmiller et al., 1999). The results of the present study indicate that the bleach and ethanol have efficiency against contamination emanating from the blending equipment used. A previous study reported that a stronger oxidation reaction was achieved when bleach was diluted in water leading to damage of organism's protein fold structure, leading to sterilization (Sana et al., 2006). Bleach also has rapid bactericidal against vegetative organisms (Fraise, 1999). Other studies demonstrated that ethanol has rapid bactericidal activity against vegetative organisms also being tuberculocidal, fungicidal and viricidal. However, ethanol has little or no activity against bacterial spores (Ayliffe et al., 1999). For difficult to sterilize materials such as instruments and seeds, a combination of both types of sterilant and often used in series.

2.5 Conclusion

The aim of the present study was to investigate the induction and proliferation of embryogenic callus tissue (ECT) in Cauliflower. It can be concluded that both plant growth regulators used and the explant type had an effect on both callus initiation and subsequent callus culture. The optimum concentration for embryogenic callus induction and subsequent culture was 2,4-D at 0.15 mg L^{-1} and Kinetin at 0.1 mg L^{-1}. It was evident that the variation in explants might be affecting callus formation. The

results showed that hypocotyl explants were superior for callus induction and subsequent culture (on both semi-solid and liquid medium) and in comparison to the other explants used, it was bright green and friable which is indicative of embryogenic potential. It was observed that the growth of embryogenic callus during subsequent culture was greater on semi-solid medium but callus tissue also appeared to have a very embryogenic behavior during subsequent culture in liquid medium. The best period for subsequent culture was 21 days.

Chapter three

Somatic embryogenesis: Induction, maturation and germination

3.1 Introduction

3.1.1 Plant regeneration through somatic embryogenesis

Somatic embryogenesis is a multi-step *in-vitro* regeneration process which starts with pro-embryogenic mass formation followed by somatic embryo formation, maturation, desiccation and plant proliferation (Von Arnold et al., 2002). This method includes the development of embryos from somatic cells which often pass through stages morphologically similar to zygotic embryogenesis (Dong and Dunstan, 1999). The developmental stages of *in-vivo* embryogenesis can be reflected by somatic embryogenesis as they pass through globular, heart and torpedo shaped stages. These embryos have the ability to develop to form normal plants in a process similar to germination but termed “conversion” (Gawel, 1989). The basic research for plant embryo development can be achieved through somatic embryogenesis (Kim et al., 2012). The use of *in-vitro* somatic embryogenesis is preferred over other *in-vitro* developmental processes such as organogenesis or axiliary bud propagation, since it can be used for micropropagation or genetic modification (Ogita et al., 2002) and for rapid proliferation of plants (Arya et al., 1993, Arya et al., 2005). Also it can be used to produce organized root and shoot axes (Mathews and Wetzstein, 1993) and in this process, a single cell or small group of somatic cells can divide and differentiate to produce an embryo (Halperin, 1966).

Two mechanisms can be followed to initiate somatic embryogenesis, either directly on explanted tissues or indirectly from unorganized callus tissues (George, 1993). The propagation procedure typically includes five steps:

1) The initiation of embryogenic cultures from explants.

2) The maintenance and proliferation of embryogenic cultures.

3) The development of embryos.

4) The maturation of embryos.

5) The germination and acclimatization and field transfer (Jain and Gupta, 2005).

3.1.2 Culture medium system

All plant species can probably achieve somatic embryogenesis when appropriate explant, culture media and environmental conditions are provided (George et al., 2008) however in practice, some species are recalcitrant. In plant tissue culture, Murashige and Skoog (MS) medium in combination with various plant hormone supplements is a universal medium used to induce somatic embryogenesis (Tanaka et al., 2000, Vasic et al., 2001, Pinto et al., 2002, Conde et al., 2004) and MS medium is most commonly used for somatic embryogenesis induction in the Brassicaceae family (Wannarat, 2009). In micropropagation, the use of liquid media is ideal for reducing the costs of plantlet production and for automation (Aitken-Christie and 1991). Uniform culture conditions can be provided by the use of liquid culture systems and the change of medium is easier and bigger vessels than those for solid culture can be used (Sumaryono et al., 2008). The liquid medium can immerse all the surfaces of the explants and therefore, nutrient adsorption can occur at all parts of the explants not only at the lower parts in contact with solid medium. There is a risk however with liquid systems that explants become oxygen starved and growth and development can be affected. Aeration and temporary immersion systems have been developed to overcome this limitation and *in-vitro* mass propagation using a temporary immersion system can be established for many plant species. Temporary immersion systems are now widely used for reducing workload and for allowing a direct contact with the medium (Etienne and Berthouly, 2002).

The use of bioreactors can play an essential role in commercial production of somatic embryogenesis and micropropagation of bud and clusters of meristems (Jain et al., 2011) and usually includes an aeration system. Bioreactors can be defined as a self-contained aseptic environment which capitalizes on liquid nutrient /air in-flow and out-flow systems. Favourable growth conditions can be provided through enabling a high degree of control over physical and chemical factors such as oxygen, pH, ethylene, carbon dioxide concentrations, temperature and aeration rate. Four categories can be used d to classify bioreactors: firstly, mechanically agitated bioreactors consisting of aeration-agitation bioreactors, rotating drums and spin-filter bioreactors; secondly, pneumatically agitated bioreactors consisting of air-lift bioreactors, bubble column and simple aeration bioreactors; thirdly, non-agitated bioreactors, consisting of gaseous phase (mist) and perfusion bioreactors; fourthly, temprorary-immersion bioreactors, consisting of systems that provide complete temporary immersion using pneumatic-driven transfer of liquid medium (RITA and TIB systems) (Etienne et al., 2006).

3.1.3. Culture medium compounds.

3.1.3.1 Plant Growth Regulators

In most species studied auxin and cytokinin can be applied as the main plant growth regulators which induce and assist the development of somatic embryos through cell division and differentiation (Feher et al., 2003).

At the induction phase of somatic embryogenesis, the PGRs used have played an important role in this process (Pacheco et al., 2007). Generally, somatic embryogenesis can be promoted by auxin alone (George, 1993b) or in combination with cytokinins (Pacheco et al., 2007). Auxin is the most important hormone as it can regulate the process of induction (Cooke et al., 1993). In many plant species, auxin

has been reported to be crucial for somatic embryo induction (Merkle et al., 1995). Although the use of 2, 4-D is most common for the induction of somatic embryogenesis, other auxins including IBA, NAA and IAA can be also used (George, 1996). Through the study of plant embryogenesis it is known that the polar transport of auxins in early globular embryos is required for the formation of bilateral symmetry (Liu et al., 1993). Somatic embryo development and morphology can be affected by the type and concentration of auxin (Al-Ramamneh, 2006) and by gradients set up across callus tissues from the cells in contact with the medium to those not in contact. Cytokinins play a role in somatic embryogenesis by promoting cell division of pre-embryogenically determined cells (Kintzios et al., 2002). The addition of cytokinins such as kinetin is often applied in the media to induce somatic embryogenesis (George, 1996).

3.1.3.2 Carbohydrates

The addition of exogenous carbohydrate to the culture medium is essential for tissues in plant cell culture (George, 1993) and carbohydrates have an important effect in promoting somatic embryogenesis (Ricci et al., 2002). It can be considered not only the source of energy and a carbon skeleton in plant but also can regulate many aspects of life activities including metabolism, assimilating partitioning and transporting, stress responses and growth and development by promoting expression of relevant genes (Koch, 1996, Smeekens, 2000, Rolland et al., 2002). Various carbohydrates are used in culture media but sucrose can be considered the most frequently used (Iraqi and Tremblay, 2001) and is a crucial medium component for the induction of embrygenesis in Brassica (Ferrie et al., 1995). It has been shown that sucrose can affect induction, maintenance and maturation of somatic embryos (Finer et al., 1989, Tremblay and Tremblay, 1991, Tremblay and

Tremblay, 1995, Iraqi and Tremblay, 2001). The osmotic potential which is provided by carbohydrate addition to the media may be important in the support of embryogenesis (Swedlund and Locy, 1993).

3.1.4 Explant type

During *in vitro* culture, variation in response can occur due to various factors such as basal medium (Zegzouti et al., 2001) and explant source (Sharma and Rajam, 1995, Haliloglu, 2002). The use of various explants for plant regeneration via somatic embryogenesis (Chee, 1992, Chee and Tricoli, 1988) and organogenesis has been optimized in some Brassica species (Cardoza and Stewart, 2004) and somatic embryos have been obtained from vegetative explants within the Brassica genus (Kirti et al., 1991, Koh and Loh, 2000). Seedling explants (cotyledon, hypocotyl and root) of commercial cauliflower genotypes have previously been used for plant regeneration (Prem, 1998) but reports of somatic embryogenesis are very few for this species. In Brassica, indirect somatic embrogenesis from hypocotyls and cotyledons explants of mustard (*B. juncea L.cv Pusa Jai kisan*) has been reported (Akmal et al., 2011). Also somatic embryos have been produced from cotyledonary explants of Chinese cabbage (*B. campestris* spp. *napus pekinensis*) (Choi et al., 1996) and from hypocotyls of oilseed rape (*B. napus L*.) (Majd et al., 2006). In terms of cauliflower (*B. oleraceae var. botrytis*), somatic embryos were produced from hypocotyls explants (Leroy et al., 2000, Raut et al., 2003) and leaf explants (Deane et al., 1997, Siong et al., 2011). The use of root explant for the first time is reported here in the present work.

3.1.5 Somatic embryo maturation, germination and conversion

The success of the regeneration method can be determined by the efficiency of somatic embryo conversion into plantlets. The survival and growth of plants from

somatic embryos *ex-vitro is* described as conversion (Bhojwani and Soh, 2001). The conversion step is crucial for the application of somatic embryogenesis in breeding and development programs (Pavlovic et al., 2012). The frequency of plant recovery is generally high from mature zygotic embryos where the maturation process is considered an important stage of embryogenic development. Embryo maturation is a culmination of the accumulation of carbohydrates, protein reserves and lipids as well as embryo dehydration accompanied by a reduction in cellular respiration (Trigiano and Gray, 1996). The subsequent germination of normal seed generally occurs in two steps: imbibition, in which the seed takes up water, and the emergence of the radicle in combination with the epi- or hypocotyl elongation. Normal germination processes include the initial elongation of the embryogenic root and visible germination occurs with the penetration of the radicle through the seed coat structures such as the testa (Bewley, 1997). Somatic embryo conversion can be defined as the development of the primary root, greening of cotyledons and hypocotyls as well as formation of a shoot apex with one or two foliar primordia (Redenbaugh et al., 1986). The maturation and germination stage of somatic embryos is usually achieved with low or zero levels of auxin (George, 1993).

3.1.6 Secondary somatic embryogenesis

The secondary somatic embryogenesis process is a special case of direct somatic embryogenesis (George et al., 2008). The emergence of such secondary embryoids apparently originate from single epidermal cells of swollen hypocotyls (Thomas et al., 1976, Loh and Ingram, 1982) and from the surface of cotyledons of primary somatic embryos. Up to about 100 secondary embryos per primary somatic embryo can be obtained (Loh and Ingram, 1982). By this phenomenon new somatic embryos can be further created from somatic embryos themselves (Vasic et al., 2001). Secondary

somatic embryogenesis systems can be used in plant breeding (Shu and Loh, 1987) since the repeated cycles of secondary embryogenesis maintain the embryogenicity for prolonged periods of time (Raemakers et al., 1995) without diminution in numbers or regeneration capacity (Shu and Loh, 1987). Secondary embryos were reported in *B. campestris* spp. *napus pekinensis* (Choi et al., 1996), *B. nigra* (Gupta et al., 1990), *B. napus* (Koh and Loh, 2000, Burbulis et al., 2007, Shu and Loh, 1987) and in *B. oleraceae* var. *botrytis* and *B. oleraceae* var. *capitata* (Pavlovic et al., 2012).

3 .1.7 Activated charcoal

Activated charcoal (AC) is a porous material composed of carbon created from wood under oxgen starved combustion. The applicability of AC in plant tissue culture medium comes from its ability for adsorption of inhibitory substances (Thomas, 2008). Charcoal has a high adsorptive capacity for gases, vapors and colloidal solids. It can be produced by destructive distillation of woods, lignite, peat, bones, vegetables, nut shells or any other carbonaceous material. Generally, activated vegetable charcoals that are produced from wood, wood waste, paper-mill waste liquors and peat are used in culture media (Pan and Staden, 1998) as it has a large internal surface area ranging from 600 to 2000 m^2 g^{-1} and pore size distributions ranging from 10 µM to 500 µM. Activated charcoal in nutrient media has an adsorption preference for moderately polar rather than highly polar or polar organics (Yam et al., 1990). Thus, aromatic compounds like phenolics and their oxidates, auxins (IAA, IBA and NAA), cytokinins (such as BA, kinetin), can have a good adsorption affinity for activated charcoal while the highly polar and readily water-soluble sugars (glucose, mannitol, sorbitol, and inositol) are not strongly adsorbed from the medium (Pan and Staden, 1998). In a wide range of plant species, the addition of AC in culture media generally promotes growth, somatic

embryogenesis and organogenesis (Nakamura and Itagaki, 1973, Ernst, 1974, Fridborg and Eriksson, 1975, Wang and Huang, 1976). It is also used to induce morphogenesis (Malhotra et al., 1998, Madhusudhanan and Rahiman, 2000, Gantait et al., 2008, Gantait et al., 2009) and it has been reported that it can be used during maturation to improve yield and quality (Groll et al., 2002, Pullman et al., 2005, Lelu-Walter et al., 2006). The inhibitor compounds of embryogenesis particularly phenylacetic acid, benzoic acid derivatives and other colorless toxic compounds can be removed or reduced by AC through adsorption (Drew, 1972, Srangsam and Kanchanapoom, 2003). In tissue culture, AC is often used for improvement of cell growth and development. Its inducer effects on morphogenesis might be mainly owing to its irreversible adsorption of inhibitory compounds in the culture medium as well as decreasing the accumulation of toxic metabolites brown exudates and phenolic exudation (Thomas, 2008). Also AC can adsorb iron chelates such as FeEGTA and FeEDDHA which have been shown to prevent the transition from globular to heart shaped embryos (Heberle-Bors, 1980). In addition, the growth inhibiting substances produced by media break down during autoclaving can be absorbed using AC (Gantait et al., 2009). There are a number of stimulatory and inhibitory activities in which AC is involved such as, the release of substances naturally present in AC which enhance growth, darkening and alteration of culture media and adsorption of vitamins, plant growth regulators and metal ions. It is believed that AC might gradually release adsorbed products like growth regulators and nutrients which become available to plants or tissue cultures (Thomas, 2008). In *in-vitro* culture the positive and negative effects of AC on growth depends on different factors, especially on the concentration of AC in the culture medium, and the species and tissue used (Fridborg and Eriksson, 1975, Ahuja, 1985, Pan and

Staden, 1998). The current study aimed to investigate and develop a protocol to produce efficient proliferation of somatic embryos from root-derived embryogenic callus tissue (RDECT) of cauliflower.

3.2. Experiments

3.2.1 The effect of temporary immersion bioreactor technique on somatic embryo induction.

3.2.1.1 Objective

The objective of this study was to investigate the ability of ECT to produce somatic embryos using the temporary immersion bioreactor technique (TIBT).

3.2.1.2 Materials and methods

The bioreactor comprised of cylindrical vessels with two compartments (500 mL each) mounted on top of each other (Fig.18). In the upper comparment, the plant material is held on a polyurethane filter and the culture medium is placed in the lower comparment. An automated air pump was connected to the container via a Millipore filter which applied pressure to the lower comparment of the container to push the medium to the upper comparment through the filter. An air vent (protected with a Millipore filter) in the lid of the container allowed the pressurized air to escape. When the air pump was switched off the liquid medium drained back to the lower compartment and the explants or callus tissues were exposed to the air again. The air pump was controlled using a timer that set the period and frequency of the liquid immersion (Jain et al., 2011). Three g of ECT were placed on the membrane in the upper comparment, SIM consisting of 0.05 mg L^{-1} IAA, 0.5 mg L^{-1} Kin and 2% sucrose were placed in the lower part. The immersion regime was for 2 min every 15 min at 25 ºC.

3.2.1.3 Results

The root-derived embryogenic callus tissue RDECT of cauliflower failed to grow when placed in the bioreactor and after 5 days all explants turned brown and died (Fig.18). This experiment was repeated three times and the same results were obtained. Therefore the second technique CI ALMT was applied in the succeeding experiments.

Figure 18. Root-derived ECT (turned brown and died) after 5 days of culture on SIM using temporary immersion bioreactor technique (TIBT).

3.2.2 The effect of explants size on somatic embryos production

3.2.2.1 Objective

To investigate the effect of sieving size class on producing somatic embryos and to determine the best size class that achieves the highest number of somatic embryos per explants.

3.2.2.2 Materials and methods

Pieces of RDECT were disrupted using a CIALMT system with a 90 s blending period. After that commercial sieves (Endecotts Ltd., London) were used to produce different explant size ranges: 300-600 µm, 600-1000 µm and 1000-2000 µm. A constant volume of 74 µL for each explant size class was used for each pot which

contained 30 mL of liquid somatic induction medium (SIM) consisting of MS medium with 0.05 mg L^{-1} IAA , 0.5 mg L^{-1} Kinetin and 2% sucrose. Pots were placed randomly on the orbital shaker used for agitation and supplemented with 16h light (spectral photo fluency 40 µmol m^{-2} s^{-2}) supplied by cool white fluorescent tubes. After 40 days the formation of SEs from RDECT was distinguished under a low power light microscope (EMZ-8TR) fitted with a camera (Infinity 2) and the number of somatic embryos was counted.

3.2.2.3 Results

According to the statistical calculation based on the mean number of somatic embryos per explants of root-derived ECT, the optimal explant size class was 600-1000 µm ($P < 0.001$) with significant differences observed between this size class and other size classes used. A good value for somatic embryo formation (30.3) was achieved from explant size 600-1000 µm while poor embryo formation was produced from explant size classes 300-600 and 1000-2000 µm (Fig.19).

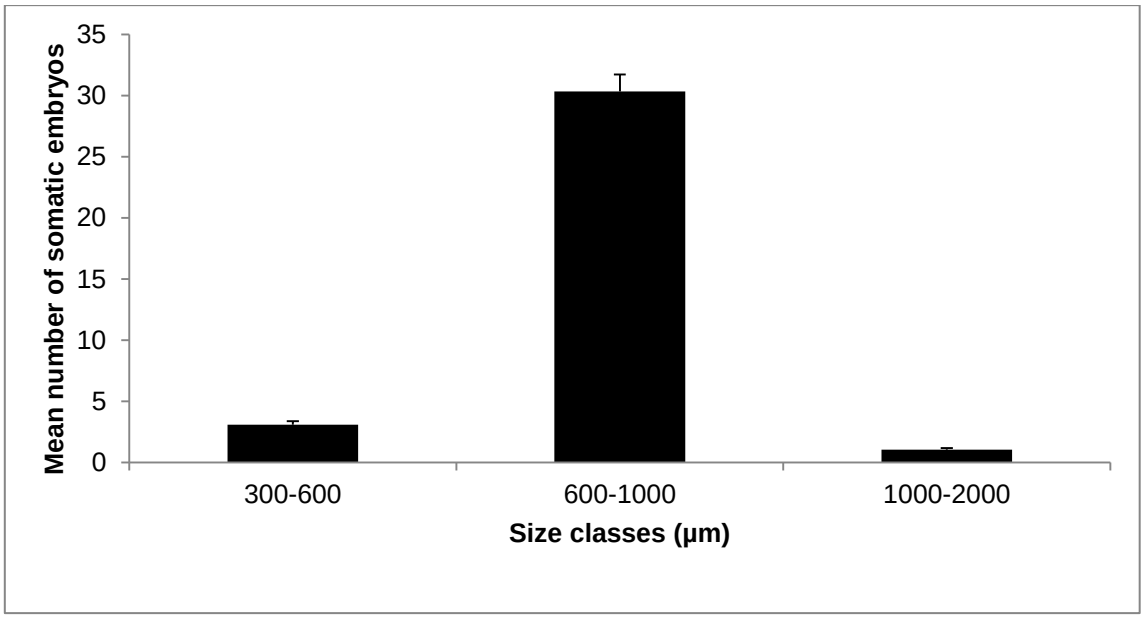


Figure 19. Effect of size classes on mean number of somatic embryos that were produced after 40 days of culture on SIM (LSD = 5.7).

3.2.3 Optimization of blending duration

3.2.3.1 Objective

The determination of the optimal blending duration to provide the highest amount of explants in a desirable size class based on fresh weight and volume of explants.

3.2.3.2 Materials and methods

Six blending durations (15, 30, 45, 60, 75, 90 sec) were applied using CI ALMT. Three g of RDECT tissue for each treatment was used. Blending was made in 50 ml of MS basal medium and the chopped ECT then separated into two size classes 600-1000 µm and 1000-2000 µm using commercial sieves (Endecotts Ltd., London).The first sieving size class (600-1000 µm) was the best for somatic embryo formation and the second sieving size class (1000-2000 µm) was previously used for successful callus culture. The total amount of explants produced from each sieving size class was recorded for each blending duration treatment based on fresh weight and volume using a 5 decimal point balance and precise volumetric measures.

3.2.3.3 Results

The results revealed that the highest amount of chopped RDECT was produced when the blending duration was 90 s in terms of fresh weight and volume for size class 600-1000 µm. There was no significant difference between this blending duration and 75 s, but it differed significantly from other blending durations. The lowest amount of explants was achieved using 15 s blending duration. For the 1000-2000 µm size class, this size produced significantly ($P < 0.001$) compared to the size class 600-1000 µm, it was observed that the highest amount of explants was obtained from a blending duration of 75 sec. It was noticed also that this treatment differed significantly from the 15 s blending duration and there were no significant

differences with all other treatments in terms of fresh weight and volume (Figs. 20 and 21).

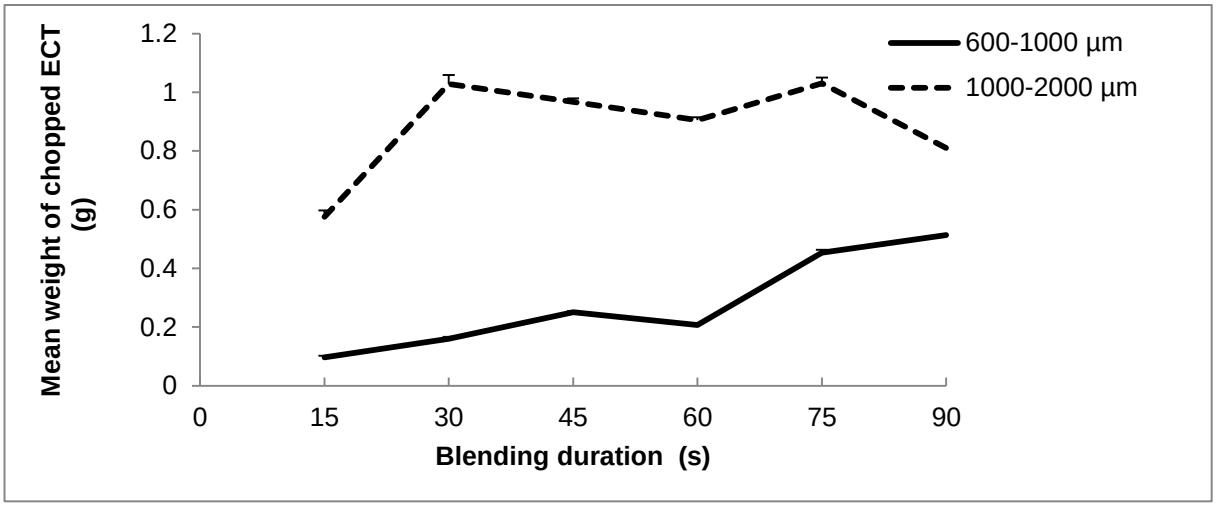


Figure 20. Effect of blending duration on mean fresh weight of chopped ECT at two size class (LSD = 0.264).

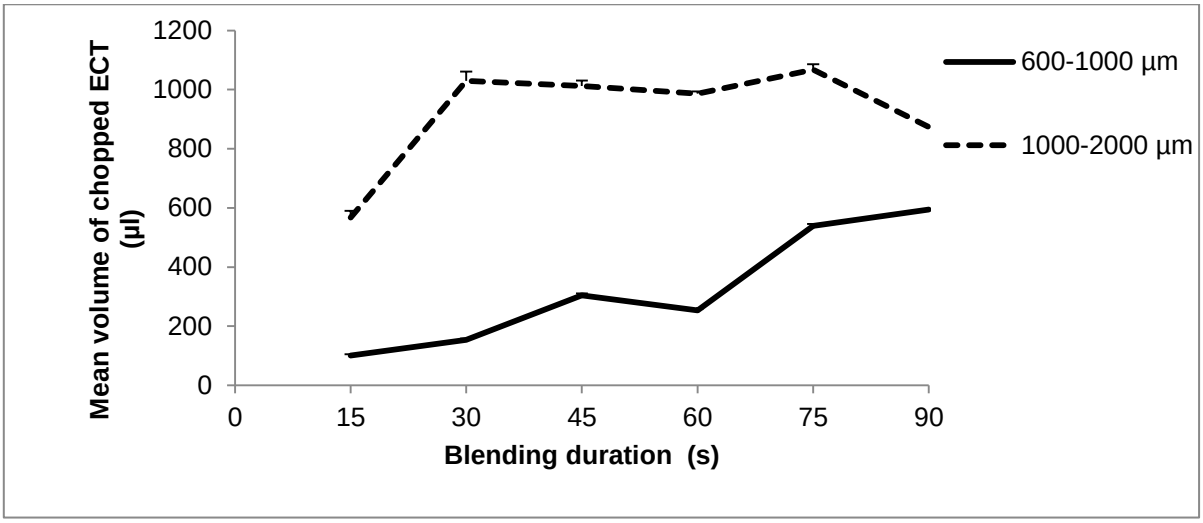


Figure 21. Effect of blending duration and size class on mean volume of chopped ECT (LSD = 265.059).

3.2.4 The effect of plant growth regulators on somatic embryos formation.

Two experiments were conducted to investigate the influence of exogenous hormones (auxin and cytokinin) used in somatic embryo induction medium (SIM).

3.2.4.1 The effect of auxin on somatic embryo formation.

3.2.4.1.1 Objective

To investigate the impact of various concentrations of auxin used in agitated liquid SIM on induction, development and maturation of somatic embryos produced from hypocotyl and root-derived ECT.

3.2.4.1. 2 Materials and methods

Pieces (3g) of hypocotyl (HDECT) or root-derived (RDECT) embryogenic callus tissue produced from four types of CIM as follows: 0.5 mg L^{-1} of 2, 4-D with 0.5, 1, 2 mg L^{-1} Kinetin and 0.15 mg L^{-1} of 2, 4-D incorporated with 0.1 mg L^{-1} Kinetin were used for somatic embryogenesis induction. Pieces were transferred to a blender containing 50 mL of MS basal medium. After 90 s as the best blending duration, commercial sieves (Endecotts Ltd., London) were used to produce explants in the size class 600-1000 µm (the optimal size for somatic embryo production) and a constant volume of 74 µL was used for each pot which contained 30 mL of somatic embryo induction medium (SIM) based on MS supplemented with Three different concentrations of IAA which were 0.01 , 0.05 , 0.1 mg L^{-1} plus 0.5 mg L^{-1} Kinetin and 2%sucrose as described by previous studies on cauliflower (Pareek and Chandra, 1978, Deane et al., 1997). The cultures were shaken at 150 rpm using a rotary shaker and incubated at 25ºC and 16 h photoperiod with a light intensity of 40 µmol m^{-2} s^{-2} supplied by cool white fluorescent tubes. Callus cultures were grown and developed on this medium for 40 days. Embryos were classified and counted in each of the 4 stages of development (globular, heart, torpedo and cotyledonary) and counted under a light of microscope.

3.2.4.1.3 Results

Somatic embryos were formed on root and hypocotyl-derived embryogenic callus explants at all auxin and cytokinin combinations. The root explants exhibited significantly more somatic embryos than hypocotyls ($P < 0.001$) (Fig. 22). Differences were noticed in both the embryogenic and organogenic potential in response to the different auxin concentrations. Throughout the first and second subcultures, cultures did not exhibit any formation of somatic embryos when ECT was transferred from semi solid (CIM) to liquid somatic embryo induction medium (SIM) where cultures formed only roots. However, after the third subculture somatic embryos and adventitious shoots were recorded. After the transfer to SIM a white friable ECT began forming on the the older yellowish explants, somatic embryos later differentiated gradually from this embryogenic mass. After 20 days (when explants became 3- 4mm in diameter), small globular structures formed on this medium (Fig. 23.1) and began elongating, then successively developed into heart and torpedo stage embryos (Fig. 23.1). The cotyledonary developmental stage was subsequently obtained after 40 days of culture on SIM with two cotyledons observed (Fig.23.2). The embryos were easily separated from the callus and shoot and root poles distinguished clearly indicating that there were no vascular connections with the mother callus tissue (Fig. 23.3). Thus, it was concluded that these structures were the results of somatic embryogenesis and were not adventitious shoots.

The RDECT explants that were produced from semi-solid CIM and then grown on liquid SIM containing 0.05 mg L^{1} IAA, 0.5 mg L^{-1} of Kinetin and 2% sucrose significantly produced the highest mean number of somatic embryos (30.9 per explant $P < 0.001$) (Fig. 24 A&B) with the highest embryogenicity rate (60%) (Fig. 25). It was demonstrated that the highest percentage 89.2% of somatic embryos at

the globular stage was obtained after 20 days. After 30 days torpedo shapes were observed on the same callus explants with a high percentage 25.1%. Cotyledonary shaped somatic embryos were obtained on explants of ECT after 40 days of culture on liquid SIM medium with percentage 62.5% ($P < 0.001$) (Fig. 26). However, during the development of somatic embryos, different cotyledon morphologies were noticed in some media used. It was observed that abnormal somatic embryos with three cotyledons were produced from both HDECT and RDECT explants and some somatic embryos with four cotyledons were produced from HDECT explants (Fig. 27). The highest mean number of abnormal embryos (those described as consisting of three or four cotyledons) was achieved on callus produced from 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin and then grown on SIM medium with 0.01 mg L^{-1} IAA and 0.5 mg L^{-1} of Kinetin (Fig. 28).

The explants of root and hypocotyl-derived ECT (Fig. 29) that were produced from semi solid CIM consisting of 0.5 mg L^{-1} 2,4-D + 0.5 mg L^{-1}Kin and then grown on medium with 0.01 mg L^{-1} IAA, 0.5 mg L^{-1} of Kinetin and 2% sucrose produced the highest mean number of shoots (Fig. 30).

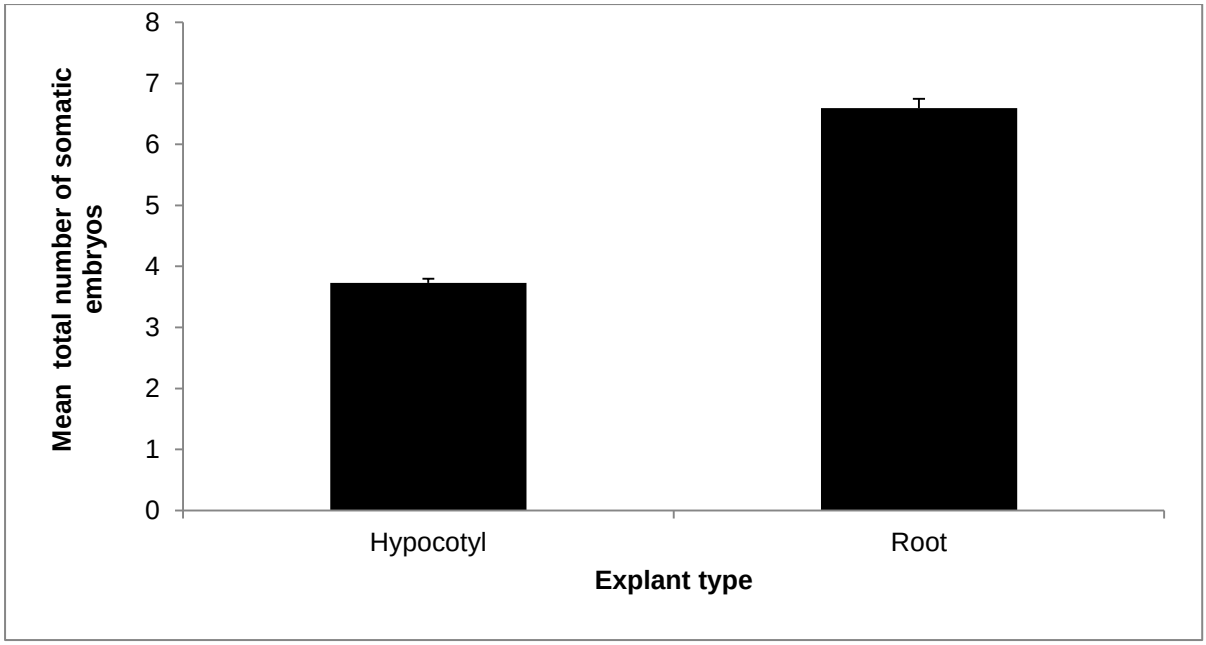


Figure 22. Effect of explant type on somatic embryos formation after 40 days from culture on SIM (LSD = 1.04).

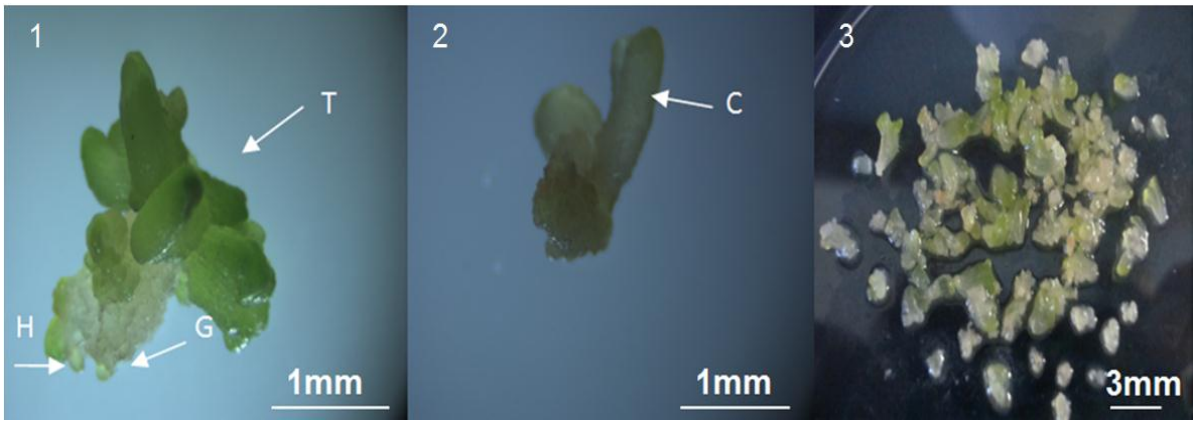
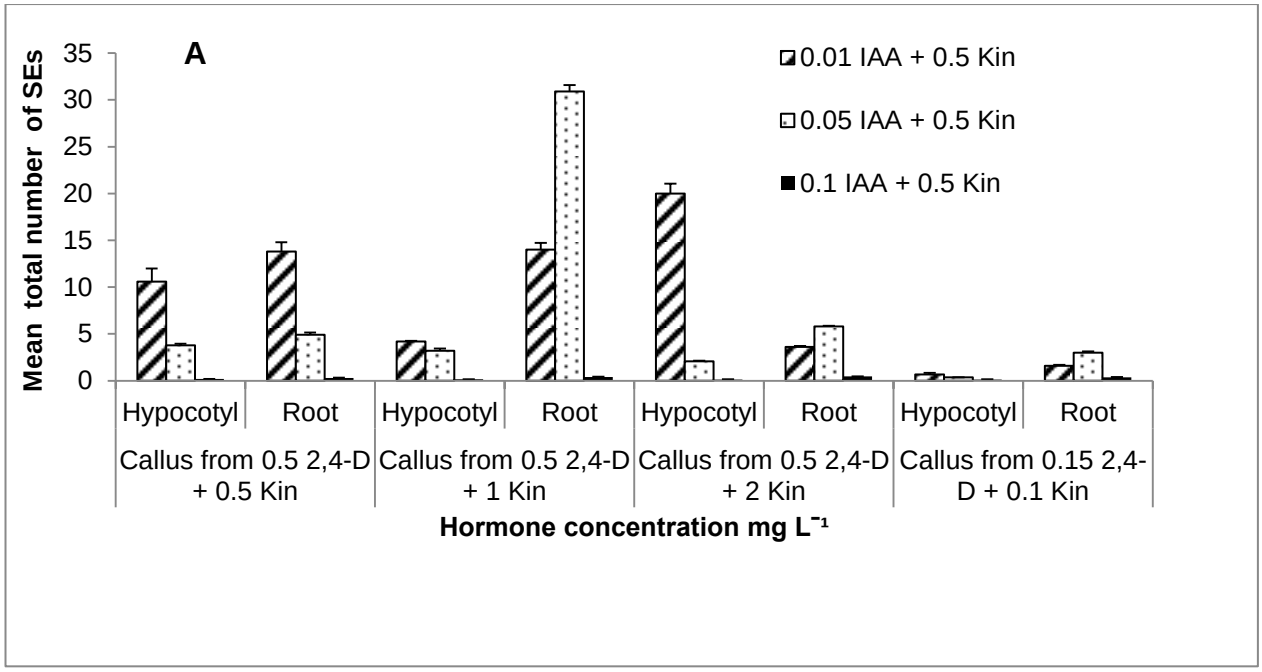


Figure 23. Effect of exogenous hormones (IAA and Kinetin) on somatic embryo production from root–derived ECT: 1) Somatic embryos at G (gloubular stage), H (heart stage) and T (torpedo stage). 2) Somatic embryo at C (cotyledonary stage). 3) Somatic embryos of different sizes.



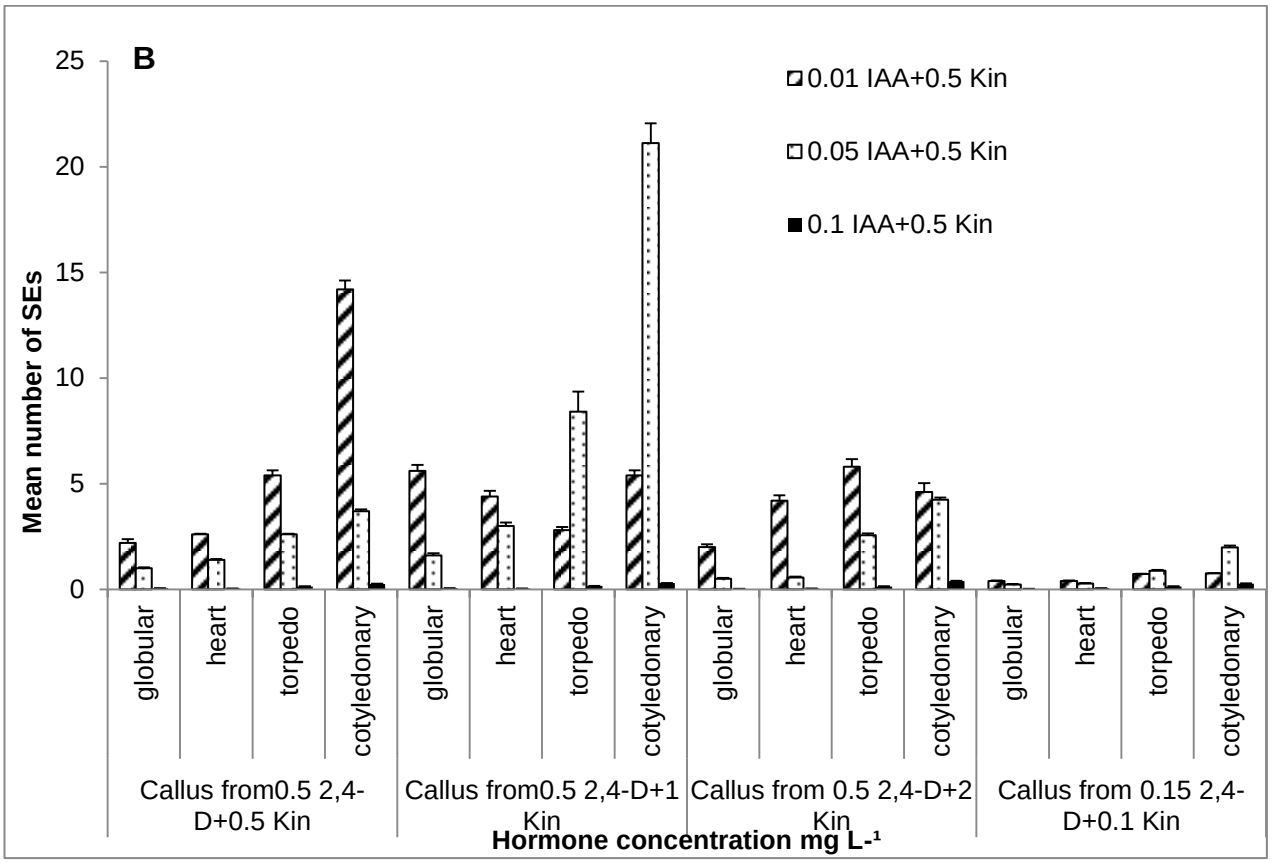


Figure 24. The interaction effect of SIM on: A) total number of somatic embryos obtained from root and hypocotyl–derived ECT produced on four types of CIM after 40 days of culture (LSD = 3.60); B) number of somatic embryos obtained at different stages of development from root and hypocotyl–derived ECT produced from four types of CIM (LSD = 4.42).

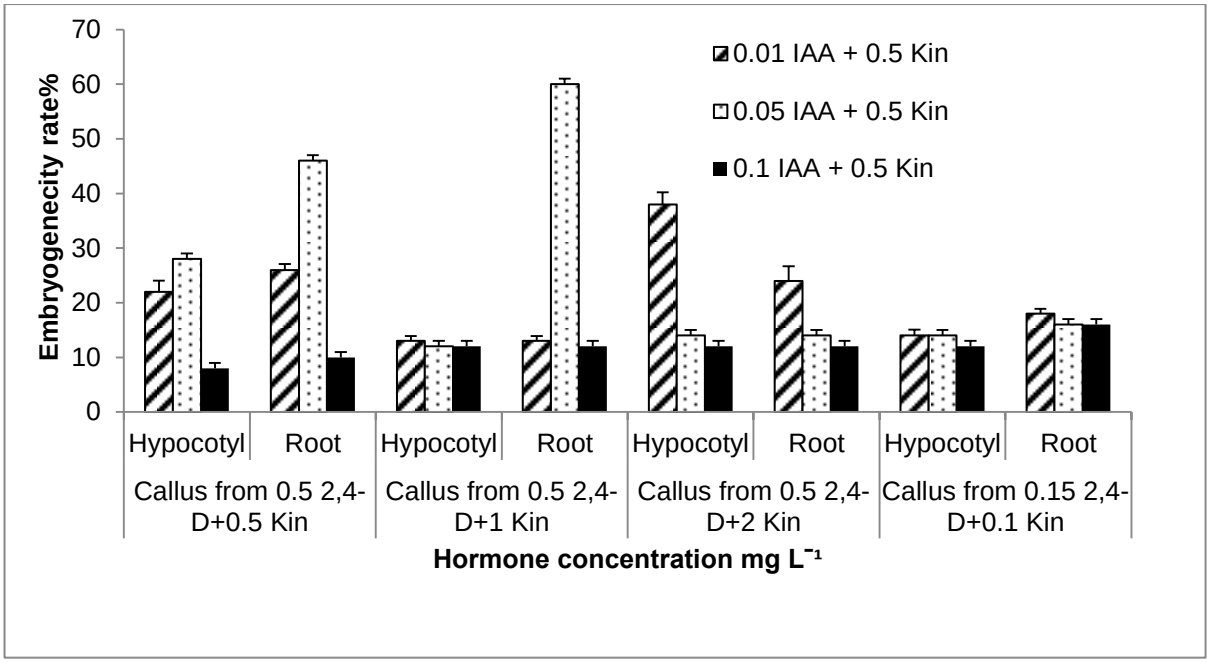


Figure 25. Effect of SIM and explant type (root and hypocotyl –derived ECT) produced from four types of CIM on embryogenecity rate % after 40 days of culture. (LSD = 8.03).

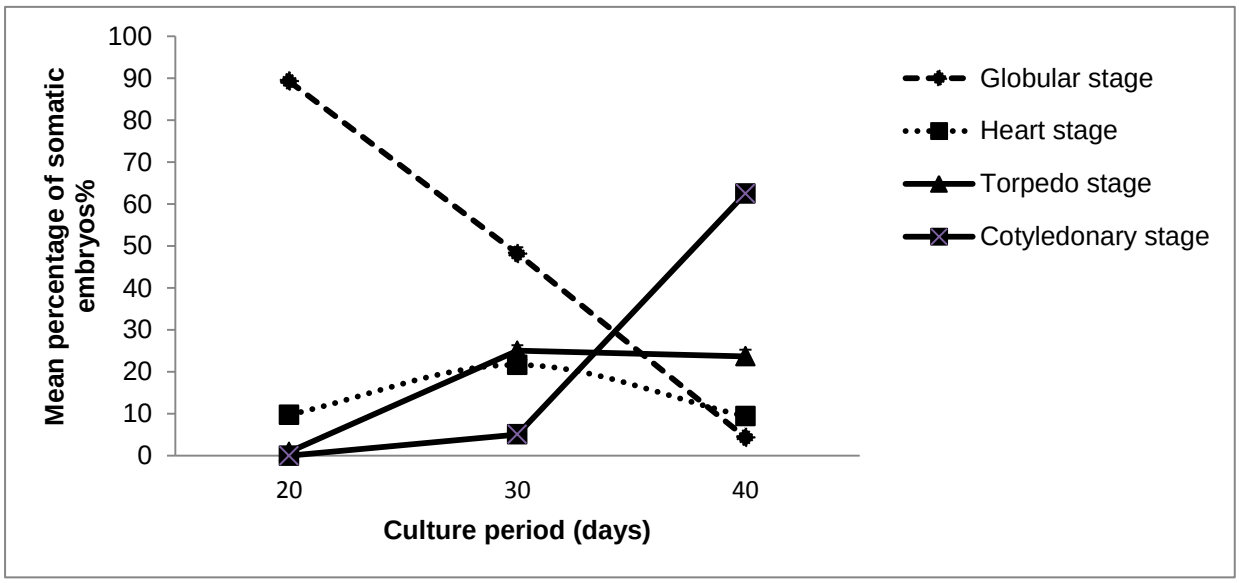


Figure 26. Effect of culture period on SIM on the percentage of somatic embryos at all developmental stages (LSD for globular stage = 6.05, for heart stage = 7.34, for torpedo stage = 8.98 and for cotyledonary stage = 6.07).

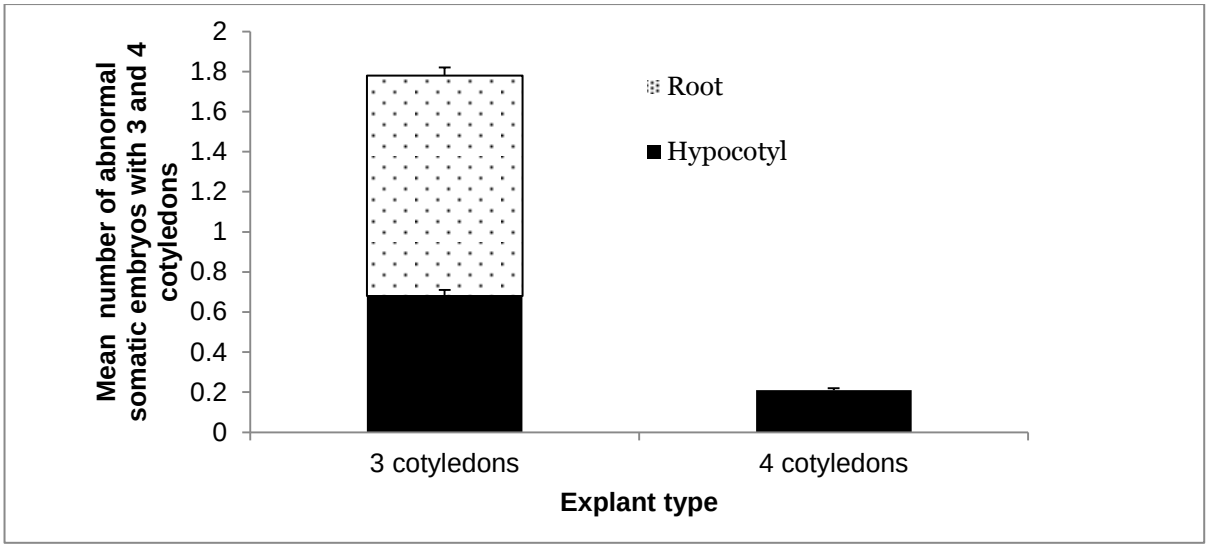


Figure 27. Effect of explant type on abnormal somatic embryo formation through culture on SIM that was supplemented with different levels of IAA and 0.5 mg L^{-1} Kin (LSD = 0.53 for 3 cotyledons and = 0.20 for 4 cotyldons).

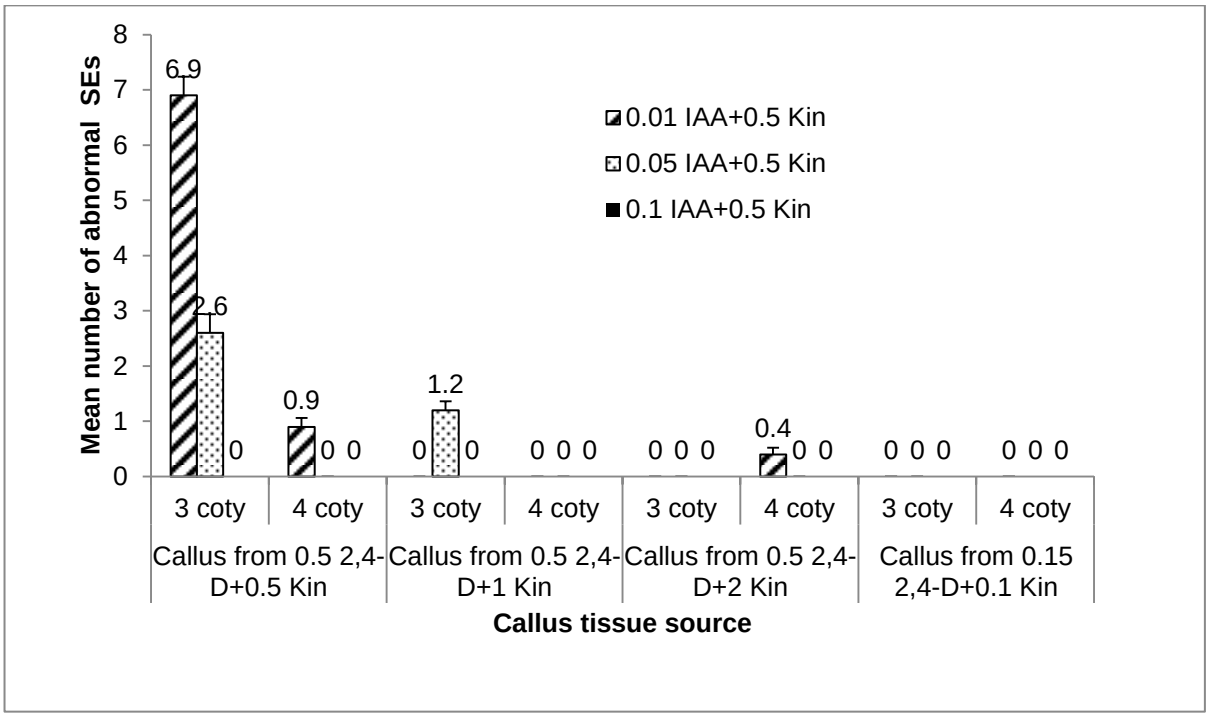


Figure 28. Effect of SIM that was supplemented with different levels of IAA and 0.5 mg L^{-1} Kin on abnormal somatic embryos produced from four types of CIM (LSD = 1.304 for 3 cotyledons and = 0.498 for 4 cotyledons).

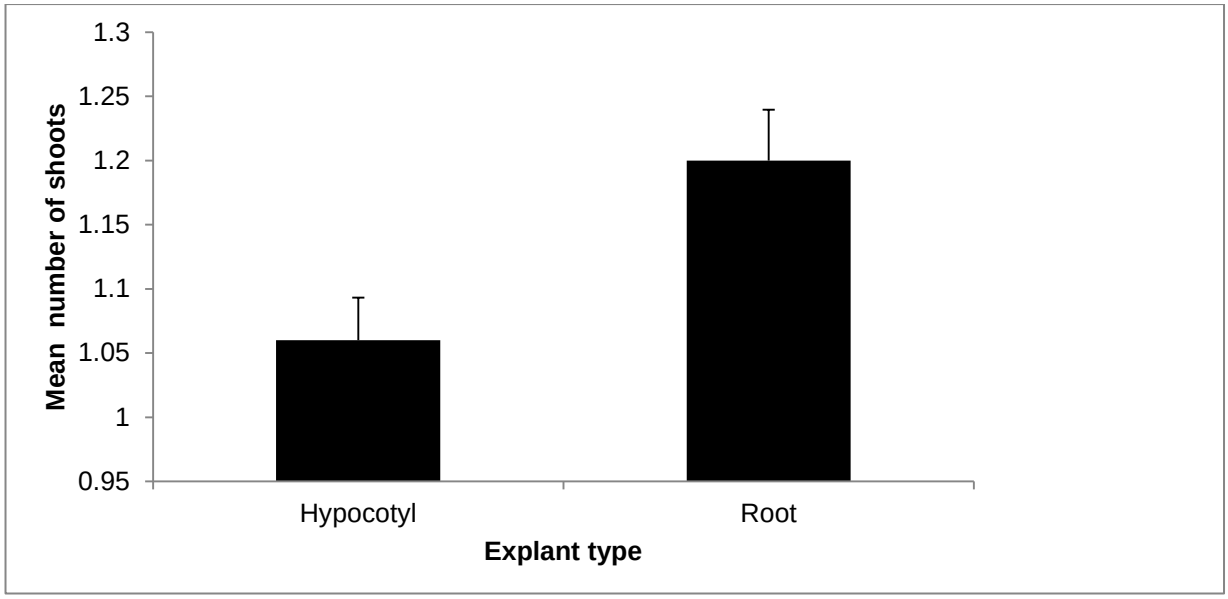


Figure 29. Effect of explant type on shoot formation from four types of CIM after 40 days from culture on SIM that was supplemented with different levels of IAA and 0.5 mg L^{-1} Kin (LSD = 1.30).

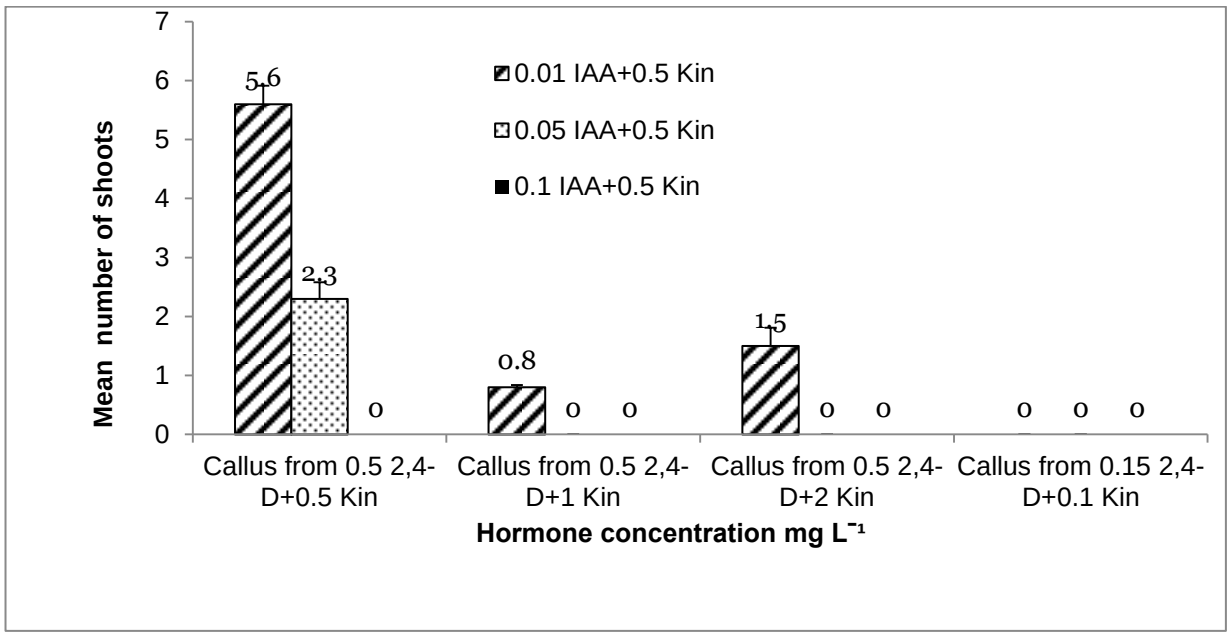


Figure 30. Effect of four types of CIM and SIM that was supplemented with different levels of IAA and 0.5 mg L^{-1} Kin on number of shoots that produced after 40 days of culture (LSD = 0.53).

3.2.4.2 The effect of cytokinin on somatic embryo formation

3.2.4.2.1 Objective

To investigate the effect of kinetin concentration on somatic embryo formation and development from root-derived embryogenic callus tissue (RDECT).

3.2.4.2.2 Materials and methods

Pieces of root–derived embryogenic callus tissue (RDECT) produced from CIM which contained 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} Kinetin (Fig. 31) were blended with 50 mL of MS medium and after 90 Sec of blending, after sieving the 600-1000 µm of explant size class was used with constant volume 74 µL per pot containing 30 mL of SIM which consisted of MS medium supplemented with various concentrations of kinetin (0.5, 1, 2) mg $L^{-1}$and IAA at 0.05 mg L^{-1} with 2% sucrose. The cultures were shaken at 150 rpm using a rotary shaker and incubated at 25 ºC and 16h photoperiod with a light intensity of 40 µmol m^{-2} s^{-2} supplied by cool white fluorescent tubes. Callus cultures were grown and developed on this medium for 40 days. A microscope was used to observe the development of embryos and the number of somatic embryos counted.

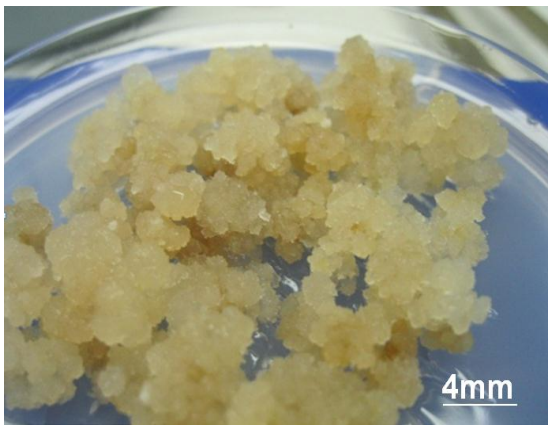


Figure 31. Root-derived embryogenic callus tissue (RDECT) on callus induction medium (CIM) through proliferation period that used for somatic embryos formation.

3.2.4.2.3 Results

The results indicated that the medium having 0.5 mg L^{-1} Kin and 0.05 mg L^{-1} IAA produced the maximum total number of somatic embryos (30.9 embryo/explants) and the highest embryogenesis rate was 60% but it did not differ significantly from the other concentrations of Kin used (Fig. 32). However, it was observed that the development of somatic embryos was better in this medium as the somatic embryos developed from globular to heart to torpedo and reached the cotyledonary stage, and the highest number of somatic embryos at the cotyledonary stage was achieved ($P < 0.001$). While a high rate of somatic embryogenesis was produced with 1 mg L^{-1} Kin plus 0.05 mg L^{-1} IAA (57%) with a mean embryo number of 27.6 embryo/explants, these stayed mostly at the globular and torpedo stages with only some conversion to the other stages. Also it was noticed that a high number of somatic embryos (28.6 embryo/explant) (Fig. 33) on the medium containing 2 mg L^{-1} Kin plus 0.05 mg L^{-1} IAA stayed at the globular stage despite an embryogenesis rate of 55 %.

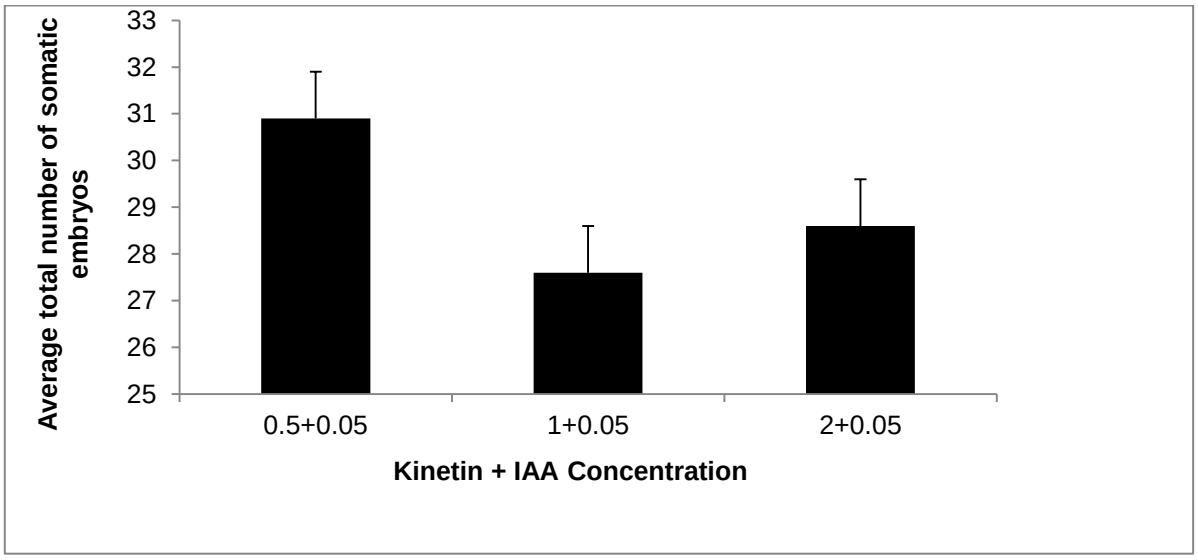


Figure 32. Effect of different levels of Kinetin concentration and 0.05 mg L^{-1} IAA on total number of somatic embryos after 40 days of culture on SIM (LSD = 8.99).

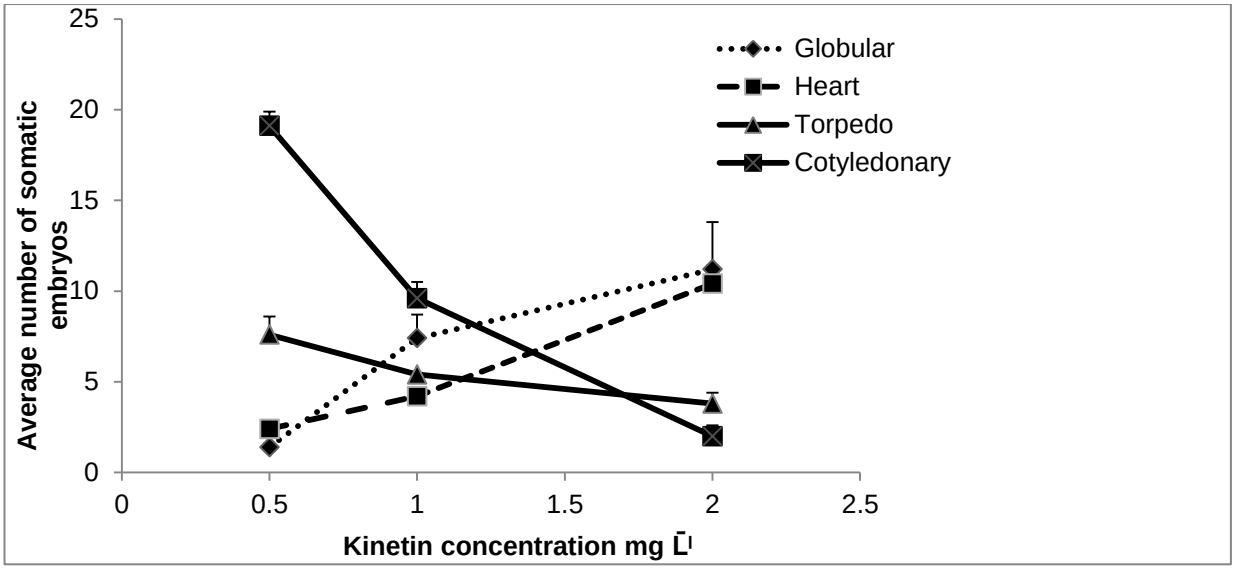


Figure 33. Effect of different levels of Kinetin concentration and 0.05 mg L^{-1} IAA on number of somatic embryos at different stages of development (globular, heart, torpedo and cotyledonary) after 40 days of culture on SIM. (LSD for globular stage = 4.93 ,for heart stage = 4.40, for torpedo stage = 4.34 and for cotyledonary stage = 5.75).

3.2.5 The effect of sucrose concentration in SIM on somatic embryos formation.

3.2.5.1 Objective

To test the effect of various types of carbohydrate and their concentrations in the culture medium on the induction rate of cauliflower somatic embryos and their subsequent development in increased osmotic potential environments.

3.2.5.2 Materials and methods

The chopped explants produced from root-derived embryogenic callus tissue using CIALMT system were placed with constant volume 74 µL in each pot which contained 30 mL of SIM consisting of 0.05 mg L^{-1} IAA plus 0.5 mg L^{-1}Kin and various concentration of sucrose and mannitol (1, 1.5, 2, 2.5, 3, 3.5 and 4%). A cryoscopic osmometer (Osmomat 030) was used to measure the osmotic potential of these liquid SIM. These cultures were agitated at 150 rpm using a rotary shaker in the laboratory at room temperature (16 hours light provided by fluorescent lights 40

µmol m^{-2} s^{-1}). Callus cultures were grown and developed on SIM for 40 days. After 20 days of culture when ECT began to produce somatic embryos, ECT was isolated from each medium and kept at -78 ℃ for 7 days, after that, the callus tissue were defrosted and extract the solutes. 50 µL (for each replicate) of callus extraction medium was used to measure the osmotic potential for ECT.

3.2.5.3 Results

Varying the sucrose concentration in the liquid SIM had a dramatic effect on somatic embryo formation from root-derived embryogenic callus tissue. The best result was achieved on the medium containing 2% sucrose. On this medium the highest number (30.2) of normal somatic embryos was obtained ($P < 0.001$). Furthermore, this concentration decreased the number of abnormal embryos consisting of four cotyledons which was significantly higher on the medium supplemented with 1% sucrose, while abnormal somatic embryos with three cotyledons occurred on media with both 1% and 2% sucrose (Fig. 34). When sucrose concentration was increased to 3, 3.5 and 4%, extreme callogenesis was observed with percentages of 85.7%, 86.4 and 80% respectively (Fig. 35) ($P < 0.001$). After 40 days of culture, the highest mean of callus diameter (4 mm) was obtained on media containing 3.5 and 4% sucrose (Fig. 36) ($P < 0.001$). The results showed that mannitol failed to induce somatic embryogenesis on any explants and it was noticed that there was no division and the cultures produced no embryos and were dead within two weeks of culture on all media with mannitol. Increasing levels of sucrose and mannitol predictably led to increasing osmotic potential in the media ($P < 0.001$) (Fig. 37). Although, the osmotic potential for ECT after 20 days (when callus tissue started to produce somatic embryos) of culture on these media was increased segnificantly (P

< 0.001) inside the callus tissue (Fig. 38), the proper osmotic environment for induction of somatic embryos can be provided by a low concentration of sucrose.

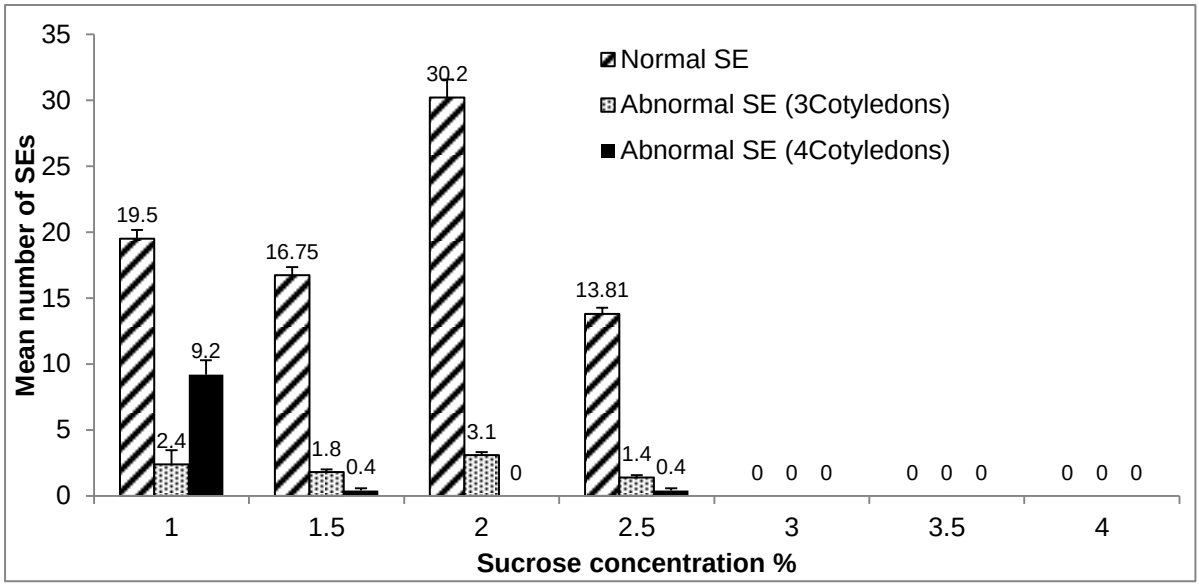


Figure 34. Effect different concentration of sucrose that added in SIM on average number of somatic embryos after 40 days of in vitro culture. (LSD = 4.12 for normal somatic embryos and 2.66 for abnormal somatic embryos with 3 cotyledons and 2.67 for abnormal somatic embryos with 4 cotyledons).

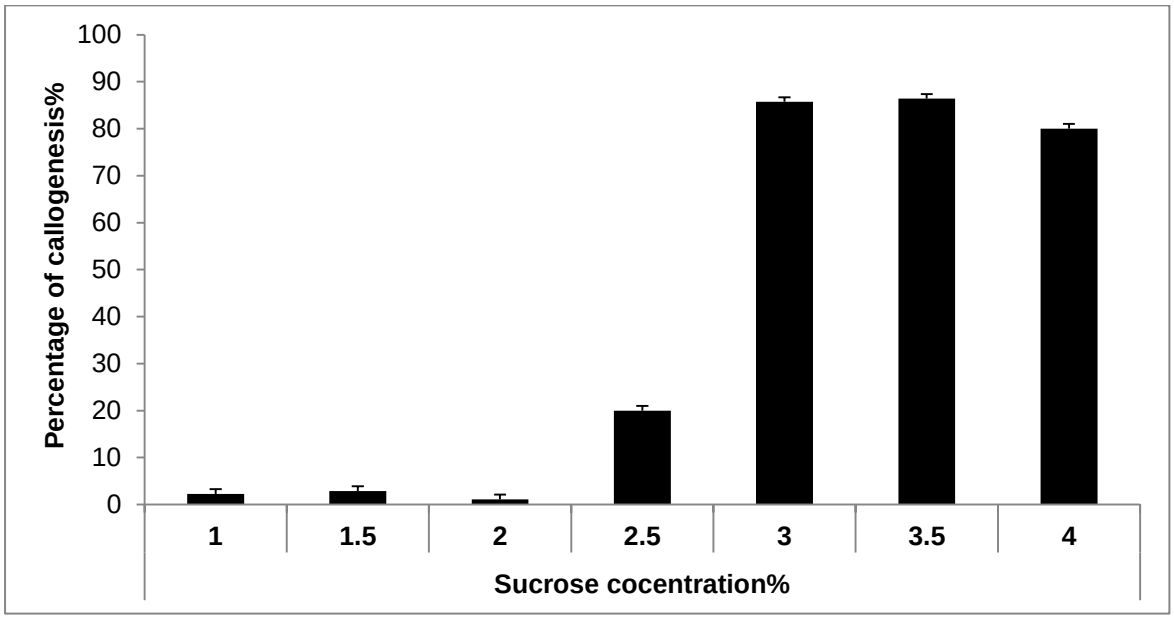


Figure 35. Effect of different concentrations of sucrose on average percentage of callogenesis after 40 days of *in vitro* culture on SIM (LSD = 7.21).

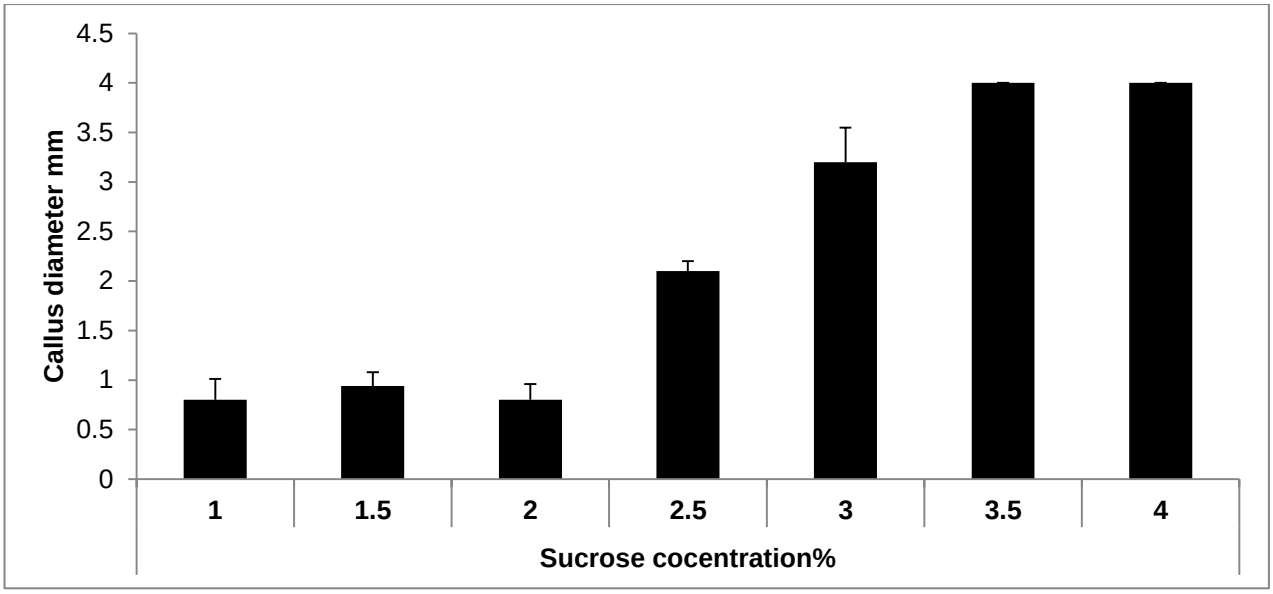


Figure 36. Effect of different concentrations of sucrose on callus diameter after 40 days of *in vitro* culture on SIM (LSD = 1.19).

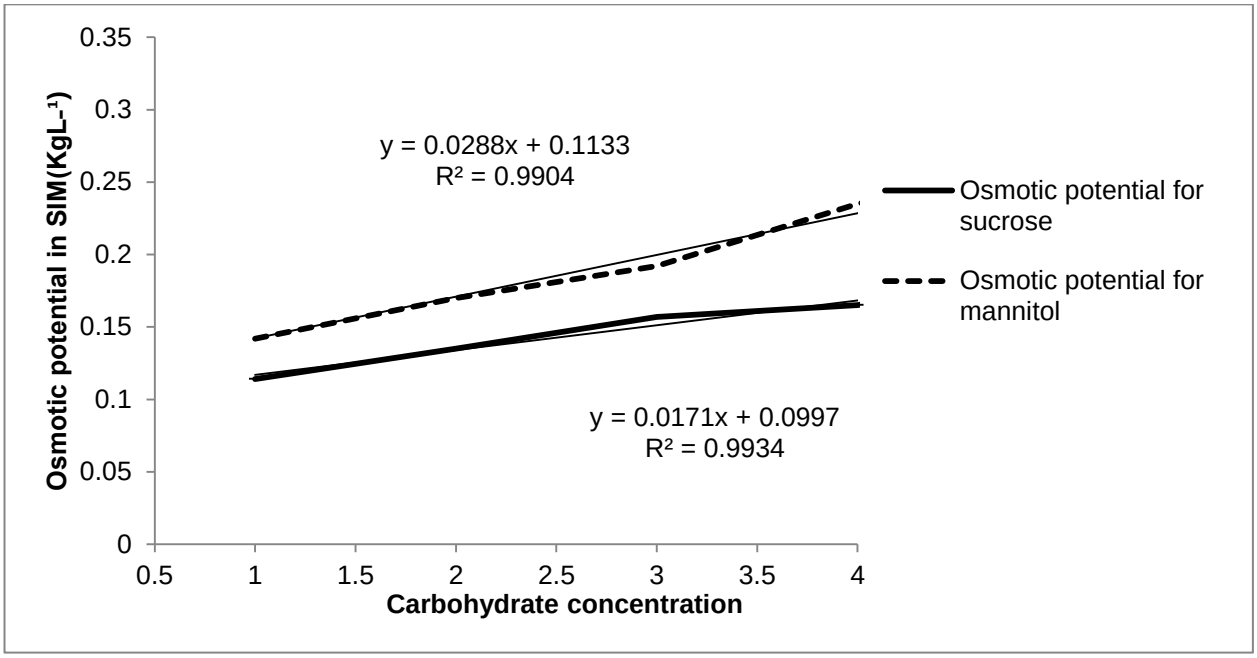


Figure 37. Osmotic potential for SIM that contain sucrose and mannitol.

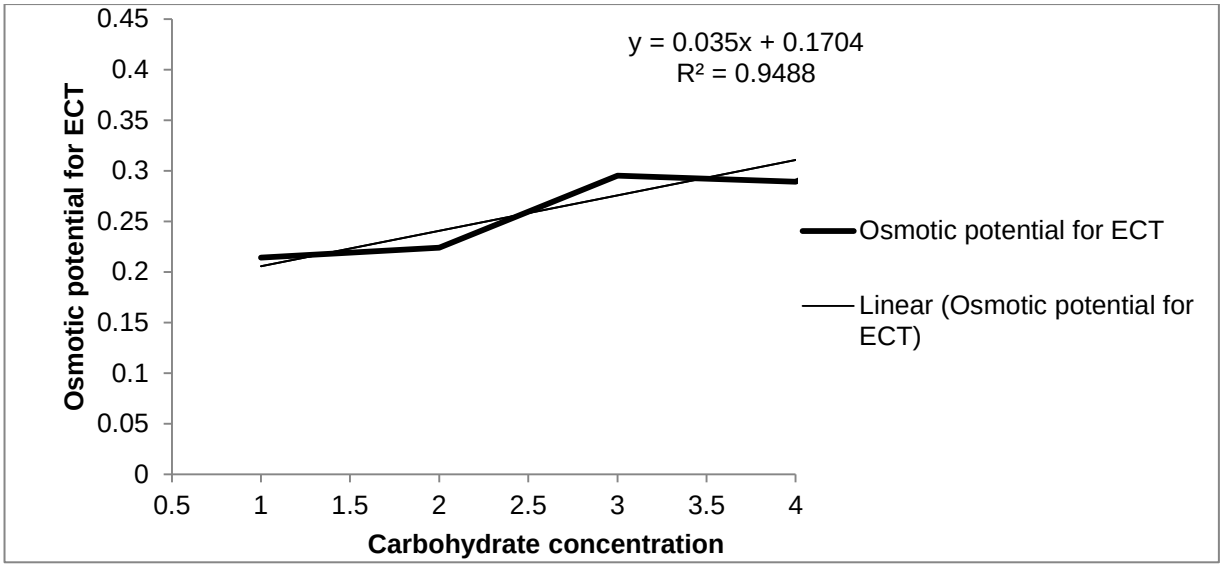


Figure 38. Osmotic potential for ECT after 20 days of culture on SIM (LSD = 0.009).

3.2.6 The effect of exogenous hormone on somatic embryos germination and conversion.

3.2.6.1 Objective

To find the most suitable culture medium that can be used to promote the germination and conversion of somatic embryos to complete plantlets.

3.2.6.2 Materials and methods

Cotyledonary somatic embryos were isolated from embryogenic clusters and transferred to the semi-solid germination media which consisted of MS medium free of growth regulators and MS enriched with 0.5, 1 and 2 mg L^{1} IBA (Indole-3-butyric acid) plus 3% sucrose and 7 g of agar. Five embryos (3-5 mm) were placed in a pot (each pot contains 30 mL of semi solid germination medium). Cultures were incubated under a 16h photoperiod at 80 µmol m^{-2} s^{-1} light intensity supplied by cool white fluorescent tubes at 22.5 ^{o}C. Conversion rate depending on germination and conversion of somatic embryos to plantlets was recorded after 40 days culture. Germination percentage was calculated as follows:-

Germination% = number of germinated somatic embryos/total number of somatic embryos * 100

3.2.6.3 Results

The results indicated that the germination and conversion of somatic embryos into plantlets did not require an exogenous supply of growth regulators in the culture medium. Thus, the highest percentage of embryo germination (in comparison with other culture media) was 60% achieved on semi-solid MS medium devoid of growth regulators after one month of culture on germination medium ($P < 0.001$). It was noticed that a healthy root elongated from the radicular end of the somatic embryo, while the cotyledonary end formed the shoot with true leaf primordia which later developed into leaves. After four weeks in culture, these germinated somatic embryos had completely converted to normal plantlets with a 100% conversion percentage on the same medium (Fig. 39).

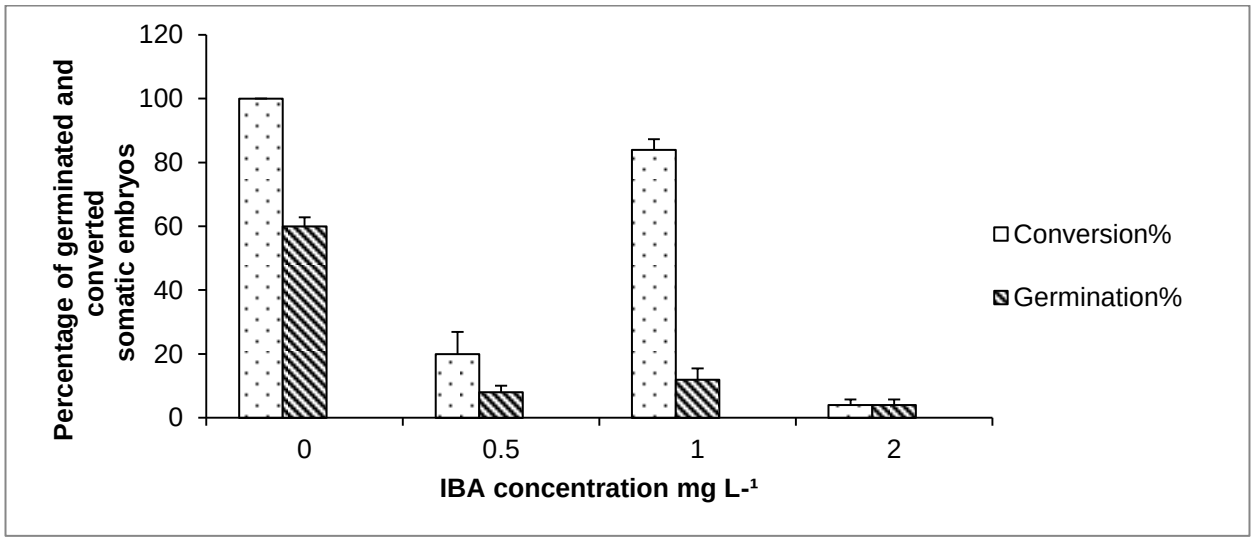


Figure 39. Effect of germination media on average percentage of germinated and converted somatic embryos (LSD = 16.20 for germination and 14.93 for conversion).

3.2.7 Secondary somatic embryogenesis induction (SSEs)

3.2.7.1 Objective

To study the ability of primary somatic embryos to produce secondary somatic embryos, qualitatively and quantitatively.

3.2.7.2 Materials and methods

Five mature primary somatic embryos (3-4mm) were isolated (used as source materials for the induction of SSEs) and placed in pots containing 30 mL of simplified medium consisting of semi-solid MS medium free of growth regulators together with MS medium with activated charcoal at three concentrations (0, 1 and 2 gL^{-1}) plus 3% sucrose . Five replicates were used for each treatment (25 primary somatic embryos for each treatment). The experiment was repeated three times. The pots were incubated under a 16h photoperiod at 80 µmol m^{-2} s^{-1} supplied by cool white fluorescent tubes and at 22.5 ^{o}C. After 60 days the production of secondary somatic embryos was recorded as the number of embryos that formed on each primary embryo and the quality of SSEs that were produced on the explants determined using observation under the low power light microscope.

3.2.7.3 Results

The use of primary somatic embryos as explants led to the formation of secondary embryos on MS basal medium free of hormones with or without activated charcoal (AC). SSEs were visible from hypocotyls of the primary SEs within 60 days of culture. It was observed that a small mass of tissue proliferated from the hypocotyls of primary embryos, and after that several SSEs emerged; different developmental stages of SSEs were noticed. However, secondary embryos developed directly on the hypocotyls of primary embryos through 60 days of culture on the same maintainance medium without subculture. Primary somatic embryos on MS basal

medium free of hormones and AC exhibited the best induction for normal SSEs (embryos with two cotyledons) as the highest mean number (9.2 embryo/explant) was achieved. There was no significant difference between treatments in terms of total number of SSEs. Two different abnormal morphological types of SSEs were observed from primary somatic embryos (Fig. 40). When AC was added to the media, it was noticed that the embryos with split collar cotyledons were obtained on MS medium plus 1 and 2 mgL^{-1} AC while abnormal SSEs with four cotyledons was achieved on MS medium. The size of embryos that formed on MS medium differed significantly ($P < 0.001$) from the MS medium with 1 and 2 mg L^{-1} AC as the highest value of embryo size (4.2 mm) was achieved. The existence of AC at 1 and 2 gL^{-1} AC led to shoot formation while, there are no shoots were noticed on medium devoid of AC. (Table.1&2).

Table 1: The effect of activated charcoal (AC) on secondary somatic embryos (SSEs) formation from hypocotyls of primary somatic embryos.

AC concentration gL^{-1}	Total number of SSEs	Number of normal SSEs	Number of abnormal SSEs(4 cotyledons)	Number of abnormal SSEs(collar cotyledons)	Number of shoots	Embryo size(mm)	Mean
0	13.2a	9.2a	4.0a	0b	0b	4.2a	5.1
1	14.2a	0.8b	0b	13.4a	1.6ab	2.0b	5.3
2	13.8a	1.2b	0b	12.6a	2.4a	1.4b	5.2
Mean	13.7	3.7	1.3	8.7	1.3	2.5	5.2

Table 2: Least Significant Difference values

Source	LSD
Total number of SSEs	6.66
Number of normal SSEs	3.34
Number of abnormal SSEs(4 cotyledons)	0.56
Number of abnormal SSEs(collar cotyledons)	5.37
Number of shoots	1.88
Embryo size	0.97

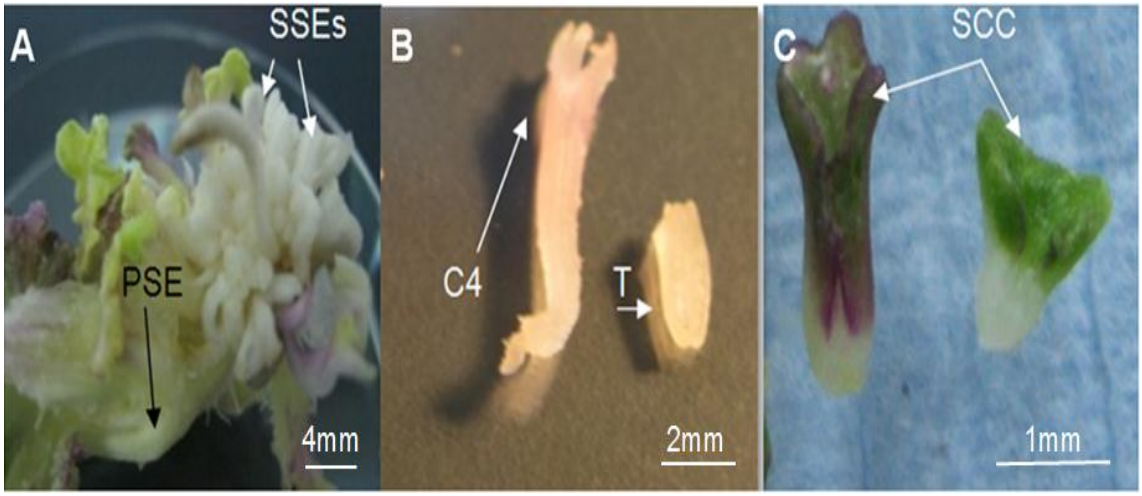


Figure 40. A) Initiation of secondary somatic embryos (SSEs) from hypocotyl region of primary somatic embryo (PSE) of cauliflower on MS basal medium. B) SSEs at torpedo stage (T) and cotyledonary stage with four cotyledons (C4). C) Abnormal SSEs with split collar cotyledons (SCC) that formed on MS basal medium with AC.

3.2.8 Secondary somatic embryo germination

3.2.8.1 Objective

To investigate the effect of SSEs initiation medium on germination rate of SSEs when transferred to germination medium.

3.2.8.2 Materials and methods

Mature and normal SSEs (1.2 mm) that were produced on MS medium with 2 gL^{-1} AC or SSEs (4.2 mm) that were produced on MS medium devoid of AC, were cultured in pots (five embryos /pot) which contain 30 mL of semi-solid germination

medium that consisting of MS basal medium supplemented with 2 mg L^{-1} of IAA. Each treatment was applied with five replicates (25 embryos for each treatment) and pots were placed in a completely random distribution inside an incubator with a 16 photoperiod at 80 µmol m^{-2} s^{-1} at 22.5 ^{o}C . The germination rate was recorded after one month from *in vitro*.

3.2.8.3 Results

The results showed that when SSEs produced from MS medium plus 2 gL^{-1} AC were cultured in a germination medium consisting of MS basal medium supplemented with IAA at 2 mgL^{-1}, embryo germination was normal, *i.e.* the cotyledons and hypocotyls began to grow slowly and the apical root axis developed (Fig. 41). The germination rate on this medium was 80%, and was significantly higher than that of the SSEs produced from MS medium devoid of AC at 22% when placed on the same germination medium after one month of *in vitro* culture (Fig. 42).

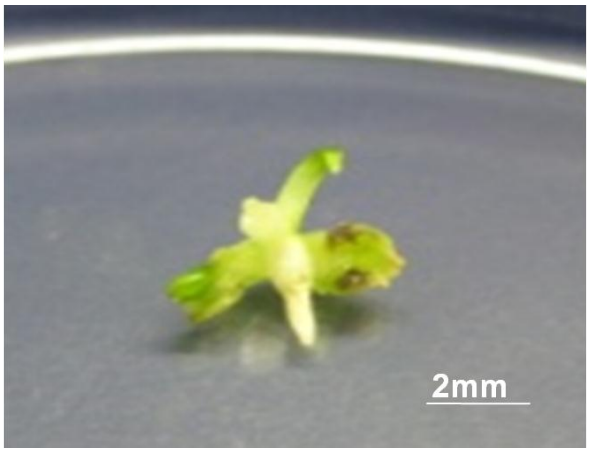


Figure 41. Germination of SSEs (produced from medium containing AC) on medium supplemented with 2 mg L^{-1} IAA.

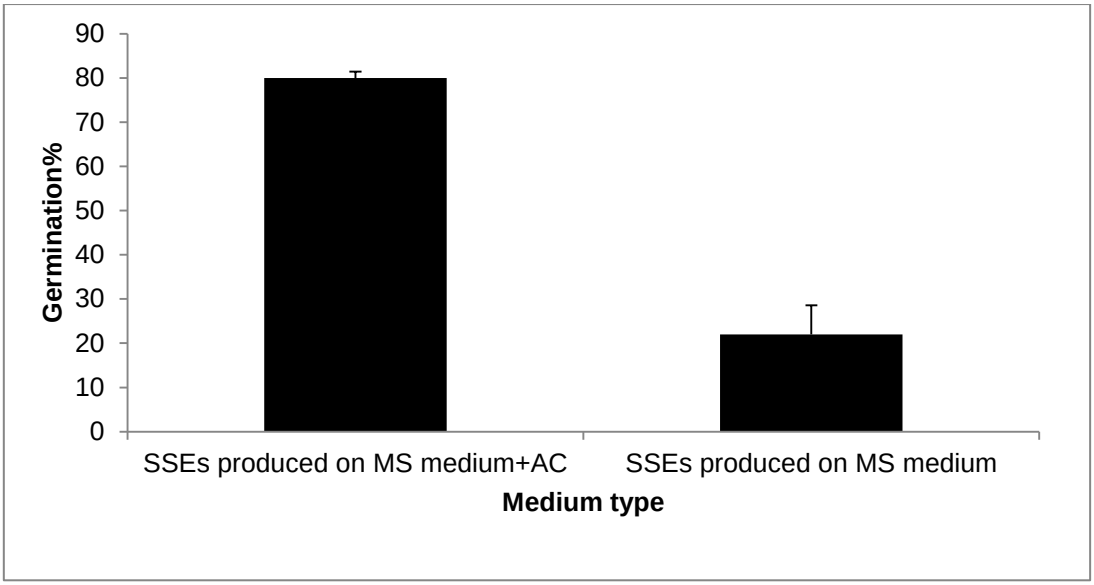


Figure 42. Germination of SSEs produced from MS medium with and without AC on medium supplemented with 2 mg L^{-1} IAA (LSD = 29.12).

3.3 Discussion

Culture system

Experiments reported in this chapter cearly indicated that an embryogenic culture technique with reliable regeneration efficiency from RDECT of an important variety of cauliflower could be established. Previously, Jain et al (1995) extolled the virtues of experimental systems to study the physiological and biochemical aspects of embryo development through somatic embryogenesis provided that recalcitrance can be overcome. The recalcitrance of cauliflower to somatic embryogenesis (Redenbaugh ,1986) has hithero limited this in studies with this species. Earlier work undertaken in the first year of the current study however demonstrated that somatic embryogenesis can be achieved in cauliflower and the current study continued to optimize this system and to investigate its suitability for bioreactor scale-up. Two different techniques for proliferation of somatic embryos in cauliflower were tested. The first one was the temporary immersion bioreactor technique TIBT. Unfortunately the callus cultures failed to develop using this technique. This observation was in accordance with a previous study reported by Jain et al (2011) in which embryogenic

calli of date palm cv.Degletbey could not grow in this system. However the use of this technique has been promoted for the development of somatic embryos of *Citrus deliciosa* (Cabasson et al., 1997), *Evea brasiliensis* (Etienne et al., 1997), *Coffea arabica* (Etienne and Berthouly, 2002), banana (Kosky et al., 2002) and oil palm (Sumaryono et al., 2008). Where it works, the TIBT system is simple and efficient. With the second technique, continuous immersion in agitated liquid media (CIALMT), somatic embryos were readily produced from explants of ECT and therefore this technique was used for all subsequent experiments.

Explant size

The direct and indirect regeneration of somatic embryos on explants depended on the size of explants. Therefore, the size of explants which are used to initiate a tissue culture must be optimized empirically (George et al., 2008). The efficient and synchronised embryo size can be obtained using a sieving process (Kreuger, 1996, Aberlenc-Bertossi et al., 1999). Although somatic embryos with all stages development (globular, heart, torpedo and cotyledonary) were produced in callus cultures from all the explants size classes that were used, the higher potential for somatic embryogenesis under the current culture condition was produced sigificantly from explant size class 600-1000 µm, as the highest numbers of somatic embryos was only achieved on it. This suggests that the morphogenetic competence of the explants has been controlled by the ability of the explant cells to re-enter the mitotic cycle. The reduced size of explants revealed synthesis of new cell wall components, such as oligosaccharides, that can be considered as signals to the cell to re-enter the mitotic cycle (Tran Thanh Van and Bui, 2000). Cells that have the potential to form somatic embryos are mitotically more active than non-embryogenic cells (Pasternak et al., 2002). In sweet potato, it was similarly observed that the decrease

in the size of cell aggregates can lead to a reduction in somatic embryo production (Chee and Cantliffe, 1989). Shigeta and Sato (1994) also have cultured horseradish embryogenic callus in MS liquid medium and after four weeks somatic embryos were significantly only obtained from a cell aggregate size of 1000 µm or less in diameter. Wannarat (2009) obtained horseradish somatic embryos from specific sized cell aggregates ranging from 1000-3000 µm in diameter whilst Chen et al (2001) reported that the smaller pieces of embryogenic calli that derived from leaflets of sexual bahiagrass which were less than or equal to 2000 µm diameter appeared uniform size and had abilities for regeneration. Jain et al (2011) mentioned that the development of date palm callus growth and improvement of synchronized somatic embryos can be achieved when embryogenic callus is finely chopped into small pieces (<380 µm). It was important here to determine the best blending duration as the amount of cauliflower explants produced for a desirable size class differed with blending durations used. It was noticed that increasing blending duration to 90 s led to the production of good amount of explants at size class 600-1000 µm. The use of a blending technique was also used effectively to produce suspension cultures from callus tissues of *Capsicum frutescens* (Williams et al., 1988) and to homogenize callus tissues (Wu et al., 2005).

Subculture effect

According to the current results and those of others (Reinert and Backs, 1968, Reinert et al., 1971), morphogenetic potential of callus tissues can be affected by the period of time during which callus cultures have been maintained. Often a short period is required to increase the regeneration capacity (one or more subcultures) and this is referred to as maturation. Thus, subculturing more than twice appears to be a prerequisite before embryogenesis is induced (Zhang et al., 2001) and this was

upheld here. The appearance in the current experiments of roots through the first and second sub-culture might be due to the high auxin concentration used which typically promotes the development of root primordium and leads to develop of root formation (Khan et al., 2006). The callus tissue which was sub-cultured in the callus proliferation phase on medium containing 0.5 mg L^{-1} 2,4-D and 1 mg L^{-1} Kinetin appeared to be a strong response to the induction of somatic embryos when subsequently transferred to liquid SIM containing 0.05 mg L^{-1} IAA and 0.5 mg L^{-1} Kinetin compared to callus produced from other CIM used. However, it seems that subculture of ECT on SIM for three times is important to induce somatic embryos from callus cultures.

Plant growth regulators

Auxin effect

Somatic embryogenesis and organogenesis can normally be triggered using auxin and cytokinin (Chaudhury and Qu, 2000, Liu et al., 2008, Jia et al., 2008). In *in-vitro* culture, various agents have been used to induce somatic embryogenesis, ranging from different plant hormones to stress treatments (Feher et al., 2003) and generally, it is thought that somatic embryogenesis can be achieved in response to modifications of various exogenous and endogenous factors such as growth regulators (Steward et al., 1964). Plant growth regulators can play an essential role in somatic embryogenesis induction (Toonen and Devries, 1996). Media containing both auxin and cytokinin have been reported to promote somatic embryo formation (Comlekcioglu et al., 2009) for many species (Majd et al., 2006). In Brassicas, the influence of auxin in combination with cytokinin on somatic embryogenesis induction was reported in several species (Pareek and Chandra, 1978, Kirti and Chopra, 1989,

Pua, 1990, Deane et al., 1997, Chamandosti et al., 2006, Majd et al., 2006, Zeynali et al., 2010, Martin and Mohanty, 2002). Two important mechanisms for *in-vitro* formation of embryogenic cells have been mentioned in the literature, asymmetric cell division and the control of cell elongation (De Jong et al., 1993, Emons, 1994) and the use of PGRs is reported to promote asymmetric cell division (Smith and Kirkorian, 1990) since that they have ability to change cell polarity by interfering with the pH gradient or the electrical field around cells (Smith and Kirkorian, 1990, Deo et al., 2010). The control of cell expansion can be associated with cell wall polysaccharides and corresponding hydrolytic enzymes (De Jong et al., 1993, Emons, 1994, Fry, 1995). The establishment of the apical-basal axis is the first step of embryonic patterning by which a smaller apical cell and a larger basal cell can be produced through an asymmetric division. The pro-embryo will be generated from the apical cell, while the basal cell can give rise to the suspensor (Mansfield and Briarty, 1991, Laux and Jurgens, 1997). The accumulation of auxin was in the apical cell of pre-globular (8-cell stage) embryos, and when the embryo became globular (32-cell stage), IAA maxima reversed to the basal part (Bowman and Floyd, 2008) towards the suspensor cells (Friml et al., 2003, Jasinski et al., 2005). That the globular embryo can initiate the establishment of polarized auxin transport in the morphological axiality was first proposed by Fry et al., (1976). In early heart–stage embryo formation a cleft forms in which the shoot apical meristem will form. After that, at a later transition stage of the embryo, the formation of cotyledons can be achieved by the transport of auxin towards the center of the cotyledonary primordia in the apical domain (Weigel and Jurgens, 2002). Thus it appears that the movement of auxin is giving orientation to the embryo (Bowman and Floyd, 2008).

The body of the plant embryo can be distinguished into four regions; cotyledons, future shoot meristem, hypocotyls and radicle during the transition from globular to heart stage (Mansfield and Briarty, 1991). The responsibility for the establishment and maintenance of embryo apical meristems can be achieved by cytokinins (Sugiyama and 1999). The activity of cytokinin can be maintained in the upper cell, while in the large basal cell, auxin can repress cytokinin activity (Muller and Sheen, 2008). During embryogenesis, the shoot apical meristem arises and can generate most of the aerial parts of a plant. The apical meristem has been subdivided into various regions, such as the central zone, peripheral zone and rib zone (Fletcher and Meyerowitz, 2000, Clark, 2001, Sablowski, 2007). The central zone is located in the center and cells division proceeds more slowly at the summit of the meristem. It can provide cells to zones, the peripheral and the rib. Lateral organs can be achieved from the surrounding peripheral zone which has a higher cell division rate. The tissues of the stem are originated from the rib zone which is located below the central zone. The leaf primordia generate from a group of cells in the peripheral zone of the shoot apical meristem.

In *Brassica rapa*, the presence of growth regulators in the basal medium led to the development of the embryo (Cao et al., 1994). Previously, Kinetin in combination with auxin, particularly IAA was used to produce somatic embryos in cauliflower (Pareek and Chandra, 1978). Auxin action could possibly be via the induction of expression of the cdc2 gene coding for the key regulatory protein kinase of the cell cycle. The accumulation of this protein in high amounts can be achieved using auxin alone but cytokinin is required for activation of the kinase (Pasternak et al., 2000). During the induction of somatic embryogenic cells, differential gene expression can lead to synthesis of mRNA and proteins and this genetic information can elicit

diverse cellular and physiological response that can result in the switching over of the developmental system of somatic cells (Archana and Paramjit, 2002).

Many histological studies on somatic embryogenesis induction have suggested that this process starts with active divisions for embryogenic callus and then developed into embryo-like structures that advance through globular, heart, torpedo and cotyledonary developmental stages (Gui et al., 1991, Alemanno et al., 1996, Quiroz-Figueroa et al., 2002, Azpeitia et al., 2003, Mandal and Gupta, 2003, Sharma and Millam, 2004). The present study showed that after 20 days of culture on SIM, 89.2% of embryos were at the globular stage, after 30 days most of these embryos had developed and converted to heart and torpedo stages. This was noticed by the decrease in percentage of globular embryo and the increase percentage of heart and torpedo embryo. After 40 days of culture, it was observed that 62.4% of embryos converted to cotyledonary stage, whereas 4.3% of the embryos stayed at the globular stage. Typically, these stages take a period of several days (Deo et al., 2010). This asynchronous development of somatic embryos is probably due to differences in culture conditions and embryo sources since the size and development stage of globular embryo is not the same (Sumaryono et al., 2000). In woody species asynchronous development of somatic embryos is very common (Tautorus and Dunstan, 1995).

The explant source

The explant source and the development stage (age) of explants can have a big effect on induction of somatic embryogenesis. Therefore, the choice of explants can be considered an important factor (Choudhury et al., 2008). The findings of the present study revealed that root derived callus is the best source for

production of somatic embryos in agreement with studies on *Brassica nigra* (Mehta et al., 1993b), *Oncidium* (Orchidaceae) (Chen and Chang, 2000) and Chinese cotton (Zhang et al., 2001) but this has never been reported before for cauliflower.

Shoot formation

The formation of shoots from callus tissue normally depends on precise environmental conditions (Burgess, 1985, Chawla, 2002). An excess of cytokinin over auxin typically leads to shoot organogenesis (Skoog and Miller, 1957, Burgess, 1985, Chawla, 2002). Cytokinin can cause the development of a single pole and the formation of a meristematic dome that can give rise to shoot primordia (Khan et al., 2006). Traditionally, cytokinins are used to promote adventitious shoot initiation and growth in micropropagation (Wannarat, 2009) and it has been shown that a deficiency of cytokinin can reduce shoot meristem size and activity (Werner et al., 2003, Higuchi et al., 2004, Werner and Schmulling, 2009). However, in the current study, the highest average number of shoots was achieved from root–derived callus on SIM medium with 0.01 mg L^{-1} IAA and 0.5 mg L^{-1} Kinetin.

Abnormalities of somatic embryos

Abnormal somatic embryo production with more than two cotyledons has been observed before (Choi et al., 1997, Griga, 2002) and was observed in the current study and was also described previously in cauliflower by Leroy et al .(2000). In general, the zygotic embryos of dicotyledonous plants have two discrete laterals to the embryo axis, whereas a great diversity in cotyledon number of somatic embryos can be shown (Soh, 1996). This might be due to prolonged time in a culture that can cause accumulation of mutations (somaclonal variations), which can lead to

morphological abnormalities such as multiplex apical formation, pluri-cotyledonary structures and fused cotyledons. The frequent initiation of new cultures and their maintenance for less than one year can cause the regeneration of phenotypically normal somatic embryos and plants (Evans et al., 1983). Different factors have been reported to affect the morphology of somatic embryogenesis such as medium composition, growth regulators, sub-culture time and frequency (Altman and Hasegawa, 2012). The subsequent culture on medium containing cytokinins can lead to the production of somatic embryos with multiple cotyledons (Lee and Soh, 1993). Embryos which have three cotyledons however can subsequently develop well and give rise to normal plantlets (Sarkar, 2009) also, Li et al. (1998) indicated that somatic embryos in caco with abnormal morphologies can develop *in vitro* into normal plants but at a slower range than somatic embryos which have normal morphologies. However, the effect of auxins on abnormalities of zygotic wheat embryos development that treated with auxin or related substances were reported by Fischer and Neuhaus, (1996).

Cytokinin effect

The requirements of auxin and cytokinin and their levels have to be determined empirically for each plant species (Jain et al., 2000). Cytokinins may have a role in cell division phase of somatic embryogenesis (Danin et al., 1993) and subsequent division of cells leads to the establishment of various forms of embryos such as globular, heart and torpedo (Akmal et al., 2011). The number of embryos in culture can be enhanced using exogenous cytokinin (Thorpe, 1995) because the initiation and maintenance of apical meristems for embryos are the responsibility of cytokinins (Sugiyama, 1999). Our findings showed that the growth and development of somatic embryos were influenced by Kinetin concentration. Globular embryos developed

into the heart and torpedo shaped embryos and converted into cotyledonary shapes faster on media containing low concentrations of Kinetin (0.5 + 0.05 mg L^{-1} IAA) while high numbers of embryos on media containing 1 or 2 mg L^{-1} Kinetin with 0.05 mgL^{-1} IAA couldn't develop to the cotyledonary stage. This may be due to high levels of cytokinin that can inhibit partially or totally the development of somatic embryo cotyledons (Ammirato, 1985).

Carbohydrate effect

The embryo quality and number can be affected by availability of carbohydrate in the medium as it is important during embryo development to fuel developmental changes (Thorpe, 1995). Sucrose has two crucial roles, it can regulate osmotic pressure and supports metabolism as a carbon source (Wan et al., 2011). Sucrose degradation can be considered as the first step for carbon utilization by cells (Hauch and Magel, 1998). Increasing osmotic potential in a medium can be achieved through hydrolyzing of sucrose into glucose and fructose (Tremblay and Tremblay, 1995, Iraqi and Tremblay, 2001) and this increase of osmotic pressure does not lead to the induction of embryo maturation or increase in the number of embryos. Similar results were obtained by Tremblay and Tremblay (1995) with spruce embryogenic tissue. The presence of sucrose at low concentration 2% was more indicative of somatic embryogenesis in *Brassica napus L.* (Ahmad et al., 2008, Majd et al., 2006) and in *Brassica junceae* L., the increase in amount of sucrose caused a reduction in somatic embryogenesis (Kirti and Chopra, 1989) and these findings are in accordance with the results presented here. Various abnormalities in embryo growth such as embryos with three or four cotyledons were noticed when low amounts of carbohydrate were used in the development medium. On the contrary, Slesak and Przywara (2003) reported that a high concentration of carbohydrate led to different

abnormalities when used in *Brassica napus* L. The high concentration of sucrose promoted callogenesis appearance on explants and this might be due to sucrose enhanced osmotic stress which has shown a significant influence on the growth of callus tissue (Javed and Ikram, 2008). George (1993) has also commented that the rate of growth, cell division or success of morphogenesis can be affected by the osmotic potential of culture media. In *Phenoix dactylifera* L. it was found that an increase in sucrose concentration from 3 to 6% suppressed callogenesis (El-Bellaj, 2000). On the other hand, mannitol is commonly used as a cell osmoticum because it can only penetrate cell walls (Rains, 1989) very slowly (Cram, 1984) and the plasmalemma can be considered as relatively impermeable to mannitol (Rains, 1989). Thus using mannitol it is possible to separate the osmoticum effect from the carbon source effect. In the current study mannitol had a very negative impact on somatic embryogenesis and a similar result was noted in *Brassica napus* L. microspore embryogenesis (Ilic-Grubor, 1998).

Maturation medium effect

Embryo maturation is a critical step in somatic embryogenesis, as the ability to form embryos and to develop these to plantlets will be dependent on this process (Leroy et al., 2000). It was noticed here that somatic embryos matured and developed on the same medium (SIM) and similar results were also obtained with mustard embryos (*Brassica juncea* L.cv. Pusa Jai kisan) (Akmal et al., 2011). The ability of SEs to develop on the same medium without transferring to another fresh medium was described previously by Sharp et al., (1980).

Germination medium of SEs

For successful micro-propagation, a strong and healthy root system on *in-vitro* derived plantlets is required (Abdul Karim and Ahmed, 2010). In general, only the basal medium without plant growth regulators is prerequisite to germinate seedling as the embryos could synthesize plant growth regulator itself (Thawaro and Te-chato, 2010). The somatic embryos that were produced here on SIM germinated well on semi-solid growth regulator free medium. This result was also observed with somatic embryos of Chinese cabbage (Choi et al., 1996), finger millet (Eapen and George, 1989), soybean (Buchheim et al., 1989, Parrott et al., 1988), peanut (Baker and Wetzstein, 1991), *Cedrela fissilis* (Villa et al., 2009), horseradish (Wannarat, 2009) and Fraser fir (Guevin and Kuby, 1992). Low germination rates of somatic embryos in many systems reported in the literature might be due to inhibitory effects of the hyperhydricity phenomenon which can cause a major problem for many different species propagated in liquid medium (Piatczak et al., 2005). Cornu and Geoffrion (1990) as well as Salajova et al. (1995) have referred to the low germination ability of somatic embryos produced by somatic embryogenesis, however in the study reported here; all embryos that germinated showed full conversion to complete plantlets.

Secondary somatic embryo induction using activated charcoal

The development of a secondary embryo is typically directly from an epidermal or sub-epidermal cell of the cotyledons or hypocotyls (Thomas et al., 1976). In the current study the appearance of secondary embryos was from hypocotyls of primary somatic embryos after 60 days of culture and this in accordance with Kumar and Shekhawat (2009) who showed that prolonged culturing leads to proliferation of secondary embryos. According the results here and other results on *Brassica nigra*

(Gupta et al., 1990), *Brassica napus* (Keller and Armstrong, 1977, Burbulis et al., 2007, Loh and Ingram, 1982, Loh and Ingram, 1983) and *Panax ginseng* (Kim et al., 2012) the production of secondary embryos was on MS medium free of growth regulators. Gupta et al (1990) had concluded that six to seven secondary embryos were obtained from *Brassica nigra* protoplast-derived embryo. Also in Chinese cabbage a few secondary embryos were formed on the surface of somatic embryos (Choi et al., 1996). Recently, Pavlovic et al., (2012) produced SSEs from the surface of hypocotyls of the cauliflower and cabbage's primary embryos when placed on PGR-free MS medium. Habituated cultures can be defined as the cultures that have ability to proliferate in culture medium without providing of exogenous growth regulators (Meins, 1989). Therefore, the primary somatic embryos when grown on MS medium free of hormone, could produce secondary somatic embryos (Al-Ramamneh, 2006). In some species the maturation of embryos does not require additional culture steps (Raemakers et al., 1995) and similarly, SSEs of cauliflower were developed and matured on the same induction medium.

The residual effect of 2, 4-D and other aromatic compounds that are produced by plant tissue which have the inhibitory effect on growth and development can be adsorbed by AC (Fridborg et al., 1987). The inhibitory effect on embryogenesis particularly by phenyl acetic acid, colorless toxic compounds and benzoic acid derivatives have been shown to be removed using AC (Drew, 1972, Srangsam and Kanchanapoom, 2003) as it has a good network of pores as well as a large inner surface area that leads to the adsorbtion of many substances (Thomas, 2008).

Somatic embryos can be classified into normal or aberrant (like morphologically abnormal in size and shape or lacking distinct stages) (Raj Bhansali et al., 1990).

Under the current working conditions, it was demonstrated that the yield of normal SSEs did not improve significantly when AC was added in to the media and a similar effect was previously reported by Aderkas et al. (2002) with somatic embryo production of hybrid larch. Gland et al., (1988) showed that AC induces desirable plant propagation from microspore culture of *Brassica napus* but it appears to have no effect on increasing embryogenesis and embryo development.

The development of abnormal somatic embryos such as the split-collar cotyledons somatic embryos can be obtained when changes in auxin distribution or activity happens during the transition from globular to heart stage. During transition, the separation of the emerging cotyledonary primordial ring into two parts occurs, and by this process bilateral symmetry is achieved around the apical-basal axis. Polar transport for auxin can play an essential role in this process as discussed earlier. The effect of auxin transport on cotyledon separation could be by two possibile routes: auxin transport might either cause the removal or accumulation of auxin in the separation region (region of the future shoot meristem), and/or too low or too high concentrations of auxin might be responsible for the lack of growth of cotyledon tissue. It can be concluded that polar transport leads to the removal of auxin from the area between the two emerging cotyledon primordial as well as a continuous transport for auxin which is a prerequisite until the separation of the cotyledon primordial can be observed morphologically. The split-collar cotyledons occur when the partial separation of the cotyledons occurs. This means that the separation process occurs asymmetrically which leads one side separating before the other and the central apical depression expanding asymmetrically across the embryo top. Therefore, it can be demonstrated that the removal of auxin begins in the central apical region of the globular or early transition embryo. After this it expands

asymmetrically across the apex of the embryo (Hadfi et al., 1998). Other researchers suggest that the internal auxin gradients which already exist in globular embryos can trigger specific steps in morphogenesis (Fry and Wangermann, 1976, Schiavone and Cooke, 1987, Michalczuk et al., 1992, Fischer and Neuhaus, 1996). In the current study the appearance of split collar cotyledons embryos was on media containing AC. Previously, has been reported that AC can adsorb auxin from culture media (Constantin et al., 1977) and perhaps excessive quantities of both exogenously supplied and tissue-produced growth regulators from medium (Karunaratne et al., 1985). Therefore, the adsorption of auxins that is released by the embryos to the medium during embryo development may interfere with morphology and germination of embryos (Merkle et al., 1995).

It is postulated that AC has affected internal auxin concentration and activity resulting in appearance of abnormal SSEs with split collar cotyledons shape. The abnormality of somatic embryos was also observed on media that contained AC on some of grapevine (*Vitis vinifera* L.) somatic embryos (Lopez-Perez et al., 2005) and on somatic embryos of *Myristica malabarica* Lam (Iyer et al., 2009). In contrast, Ćalić-Dragosavac et al., (2010) reported that the addition of AC in the maturation and conversion medium of horse chestnut (*Aesculus hippocastanum* L.) androgenic embryos led to a reduction in the percentage of abnormal structures. According to the findings presented here, the SSEs that formed on media enriched with AC appeared morphologicaly smaller in size (1.4 mm) compared with those originated on MS medium devoid of AC. Merkle et al. (1995) reported that the accumulation of storage products and cellular expansion which happen during embryo maturation result in an increase the embryo size. Thus the variation in embryo length can be considered a good marker for the maturation process (Corredoira et al., 2003).

However, in contrast, Pintos et al. (2010) referred to a significant increase in a relative size of cork oak somatic embryos that can be enhanced using AC in the medium as embryos size increased to 1.3 cm in length. A compromise between the number and quality of SEs developed and the use of AC is always complex and remains questionable but clearly needs to be optimized for each species in turn (Lelu-Walter and Paques, 2009).

The adventitious shoot proliferation from tissue cultured explants can be affected by many factors such as culture medium (consisting of growth regulators and their combinations), genotype, physical environment, explant developmental stage (Qu et al., 2000) .Therefore, another aspect of the current study was the development of shoots by direct organogenesis from hypocotyls of primary somatic embryos on media enriched with AC. Previously, it was reported that a stimulative effect on the growth and organogenesis of different plants is achieved when AC is applied in the medium (Mensuali-Sodi et al., 1993). Similarly, Nayanakantha et al., (2010) found that the addition of AC to MS medium led to induce shoot multiplication of *Aloe vera.*

Secondary somatic embryo germination

As it was shown here the SSEs that were produced on medium containing AC could give a good germination rate when placed on germination medium supplied with 2 mg of IAA. Various *in vitro* factors can affect maturation and germination of somatic embryos such as sugar and auxin concentration (Al-Khayri, 2003, Al-Khateeb, 2008). Surathran et al (2011) have stated that the initial ability for germination can be ensured by the presence of AC in the medium, while including plant growth regulators is essential for the further development of the embryo. The positive effect of AC on development of root growth of plantlets was reported by Kim et al., (2012) on SSEs plantlets of *Panax ginseng* Meyer, by Sarma and Rogers (2000) on

plantlets of *Juncus effuses* L. and by Zhou and Brown, (2006) on SEs plantlets of North American ginseng.

3.4 Conclusion

A reliable method was developed to produce primary and secondary somatic embryos from RDECT of cauliflower. Following this breakthrough a protocol was developed for the mass production of somatic embryos using a bioreactor system. The optimization of culture conditions for induction, development, germination and conversion of primary somatic embryos to plantlets is described. It was clear that the use of the CIALMT technique could be a very efficient tool for the high proliferation of primary somatic embryos of cauliflower. The effect of exogenous growth regulators and sucrose concentration as well as explant size and their source on primary somatic embryo formation was evaluated and optimized. The effect of AC on SSEs formation and germination was evaluated.

Chapter four

Encapsulation of somatic embryos for artificial seed production

4.1 Introduction

4.1.1 Artificial seeds production via somatic embryos

The rapid development of somatic embryogenesis procedures has led to the use of somatic embryos (SEs) as artificial seeds for plant micropropagation (Vicient and Martinez, 1998, Helal, 2011). Artificial seed technology can be considered as one of the most important applications of SEs (Fujii et al., 1987). The current definition of artificial seed is an artificially encapsulated somatic embryo, shoot or any other meristematic tissue (Jain and Gupta, 2005) which can behave like true seeds and develop into seedlings (Nor et al., 2011) under *in vitro* or *in vivo* conditions (Jain and Gupta, 2005) and thereby can eliminate the acclimation steps necessary in micropropagation and give breeders greater flexibility through using this technique (Onishi et al., 1994). Somatic embryos can be characterized by the absence of any protection, a high water content and a very low reserve level (Kersulec et al., 1993). The artificial seed technique includes the encapsulation of tissue culture derived somatic embryos in a protective coating (Rao et al., 1998).

Plant species that produce non-viable seeds face difficulties to regenerate and use other propagation methods such as vegetative propagation, artificial seeds can be applied as an alternative method for such species (Daud et al., 2008). Somatic embryos have both shoot and root apical meristems resembling zygotic embryos but they do not have seed coats (testa) (Kysely and Jacobsen, 1990) and encapsulation of somatic embryos can be applied to produce an analogue to true seeds. Normally somatic embryos are mixed with an encapsulation matrix in order to provide protection to the embryos (Chawla, 2002) and water uptake and nutrient release can be controlled by the encapsulation matrix (Senaratna, 1992). A suitable formulation of the medium in the coating complex of encapsulated embryos is requisite to

promote the germination frequency and subsequent conversion to *in-vivo* plantlets (Jain and Gupta, 2005).

Many beneficial advantages can be offered through the use of artificial seed technology in commercial situations for the regeneration of a variety of crops at low cost (Jain and Gupta, 2005, Reddy et al., 2012). The potential for long term storage whilst maintaining viability and stability during handling is also highly advantageous (Ghosh and Sen, 1994, Helal, 2011). Also, artificial seeds can reduce the size of propagules (Chand and Singh, 2004) and be transported and planted directly from *in vitro* to field conditions (Ghosh and Sen, 1994, Helal, 2011). With many natural and improved genotypes seeds are genetically heterogeneous, and large amounts of time are needed for the production of homogeneous genotypes. Plant propagules with the same genes as the mother plant (clones) can be obtained simply when embryos are achieved through somatic embryogenesis (Latif et al., 2007). Commercialy, the production of artificial seeds requires overcoming two research hurdles: 1) encapsulation matrix development; 2) production of somatic embryos with high quality which have characteristics morphologically analogous to a zygotic embryo. Phenotypically, high quality somatic embryos will produce plants analogous to the mother plant (Redenbaugh et al., 1986).

4.1. 2 Encapsulation techniques

Encapsulation can be regarded to be the best technique to protect and convert *in-vitro* derived propagules into artificial seeds (McKersie et al., 1993). Cryo-protectant materials such as alginate gel, hydrogel , ethylene glycol and dimethylsulfoxide (DMSO) and others that have ability to develop a coating that can protect explants from the mechanical damage that can occur during handling (Harikrishna and Ong, 2002) by supplying rigidity to the explants (Winkelmann et al., 2004) yet permitting

germination and conversion without promoting undesirable variations (Harikrishna and Ong, 2002). The choice of the hydrogel that is used for encapsulation of somatic embryos is important. In many plant species the vegetative propagules can be encapsulated in calcium alginate beads (Pattnaik and Chand, 2000, Brischia et al., 2002, Danso and Ford-Lloyd, 2003, Chand and Singh, 2004, Singh et al., 2006a, Singh et al., 2006b). Alginate dissolves easily and is stable at room temperature (Redenbaugh et al., 1993), and produces a permeable gel with $CaCl_2.2H_2O$ (Datta et al., 1999) with moderate viscosity and low spinnability of solution and it has low cost and low toxicity for encapsulated explants (Saiprasad, 2001). The use of alginate as a protective coating for somatic embryos was initially reported by Redenbaugh et al (1984) and Redenbaugh et al. (1986) reported the encapsulation fo cauliflower somatic embryos in alginate, but difficulties in producing large numbers of embryos meant that this research did not continue.

4.1.3 Artificial seed endosperm

The ultimate viability of the artificial seeds can be affected by the matrix material or simulated endosperm, as the matrix is responsible for the immediate surrounding of the plant materials. The temporal and quantitative supplement of growth regulators and nutrients along with an optimal physical environment can determine the quality of artificial seeds (Senaratna, 1992, Khor and Loh, 2005). Artificial seed can also be used as a carrier for micro-organisms, nutrients, antibiotics, plant growth regulators, pesticides and fungicides (Saiprasad, 2001). Also it not only provides the physical protection for embryos (Gray, 1989) but the carbon source (Antonietta et al., 1998) and growth regulators to control and sustain growth through germination (Nieves et al., 1998, Antonietta et al., 1998). The endosperm of artificial seed could be similar to the endosperm of seeds but can also be manipulated so as to control growth and to

reduce the difficulties of the germination of somatic embryos (Castillo et al., 1998, Kumar et al., 2004, Malabadi and Van Staden, 2005). The aim was to study the possibility of using somatic embryos for producing artificial seed and to investigate the best encapsulation matrix as well as the methods used to establish an efficient encapsulation protocol.

4.2. General materials and methods

4.2.1. Explant preparation

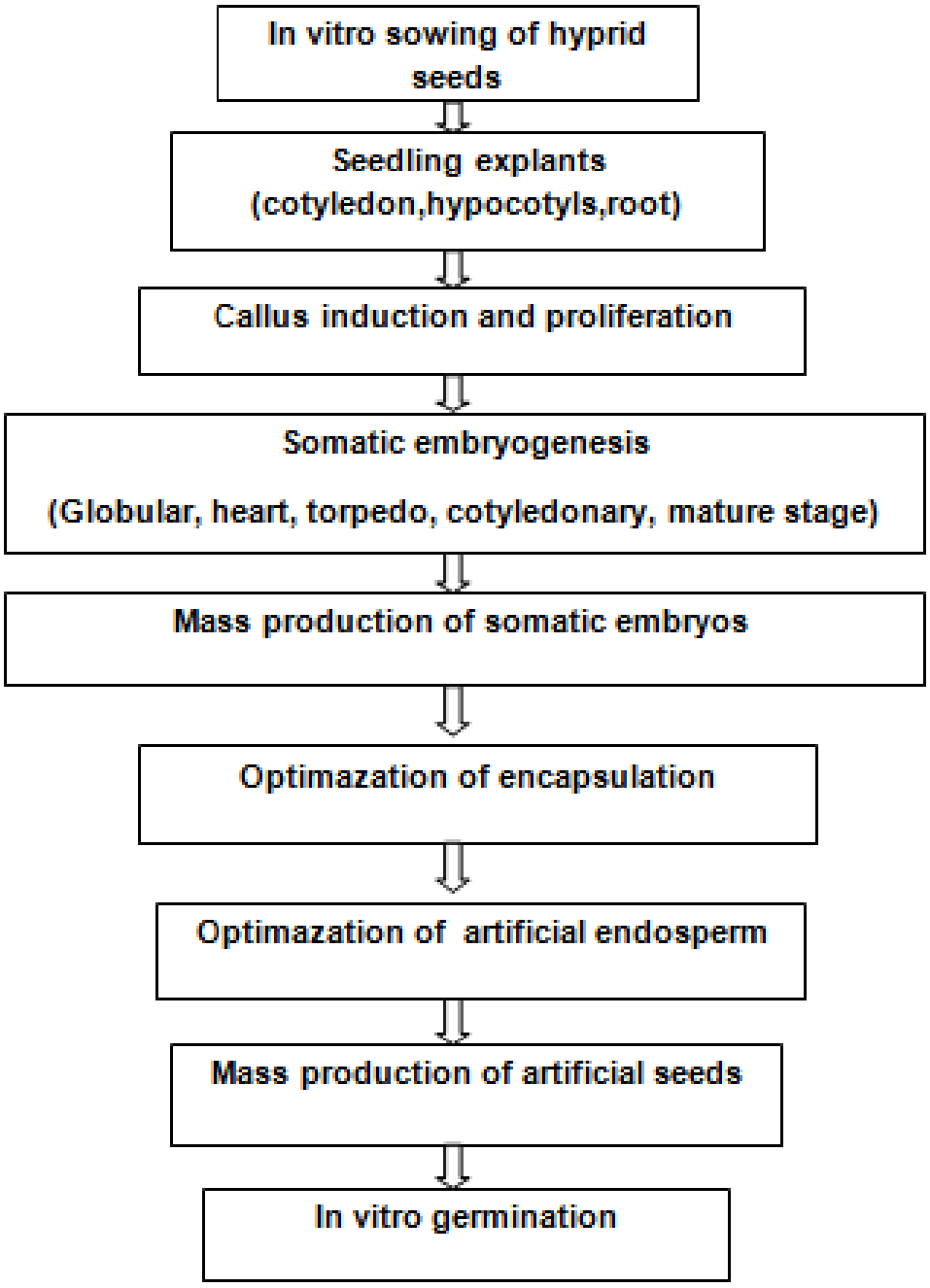
The explants of root-derived ECT produced using CIALM technique were placed on agitated liquid SIM (74 µL for each pot which contains 30 mL of SIM). After 40 days of culture, 3-4 mm somatic embryos were picked from the callus cultures and used as explants to produce artificial seeds.

4.2.2 Encapsulation matrix preparation and bead formation

An efficient encapsulation of somatic embryos is a prequisite for artificial seed production (Maqsood et al., 2012) and the hydro-gel encapsulation procedure developed by Redenbaugh et al (1987) was the most suitable method for the preparation of artificial seed. In this procedure, sodium alginate (Na-alginate) was prepared by mixing with calcium free liquid MS medium. The explants were immersed in Na-alginate solution and then dropped into calcium chloride solution. In the current study, the procedure was applied with some modifications. The encapsulation matrix was made using various concentrations of sodium alginate (2, 2.5, 3% w/v) (Sigma Ltd) in MS medium with 30g L^{-1} sucrose. The Na-alginate solutions were prepared with distilled water and stirred continuously up to 30 min on a magnetic stirrer until the solution became viscous. This was followed by sterilization by autoclaving at 1 bar, 121°C for 15 minutes but it was noticed that the

high temperatures reduced its gelling ability. Therefore, Na-alginate solutions were sterilized using the Tyndallisation procedure described by Rihan, (2013) as follows: 1) Heat at 80°C for 15 minutes to kill most micro-organisms, but not spores; 2) Rest at room temperature for five hours to allow spores to germinate; 3) Heat at 90°C for 15 minutes to kill germinated spores; 4) Leave overnight and heat at 90°C for 15 minutes (as insurance). Also, Calcium Chloride ($CaCl_2$. $2H_2O$) solution was prepared in concentrations of of 5, 10, 15 g L^{-1} (34, 68 and 100 mM) which were evaluated for complexation (an ion exchange reaction occurs in 20 min between Ca and Na leading to the creation of insoluble calcium alginate). Calcium chloride solutions were sterilized by autoclave (1 bar, 121°C for 15 minutes). The explants (3-4 mm somatic embryos) were mixed well with the sodium alginate solution inside small plastic pots by the gentle shaking. The explants were dropped into the calcium chloride solution using pipettes trimmed to give 2-4 mm holes to give a single explant in each bead.The beads were kept in the Calcium chloride solution for 20 min for polymerization. After that, the beads were collected with a sieve and washed three times using autoclaved distilled water under aseptic conditions in a laminar flow chamber in order to remove all residual Calcium Chloride. After bead hardening, the charecteristics of beads were recorded, then the artificial seeds were cultivated on a basal MS medium free of growth regulators with 30g L^{-1} sucrose and 7 g L^{-1} agar for one month and seed survival (assessed as any artificial seed manifesting new tissue growth) was recorded to determine the optimal concentration of Na-alginate and Calcium Chloridefor encapsulation.

4.2.3 Summary of artificial seed production procedure in cauliflower.



4.3 Experiments

4.3.1 Optimization of somatic embryo encapsulation

4.3.1.1 Objective

The objective of this experiment was to determine the optimal concentration of sodium alginate and Calcium Chloride solution for the encapsulation matrix to produce ideal beads of cauliflower somatic embryos.

4.3.1.2 Materials and methods

For encapsulation purposes, three concentrations (2.0, 2.5 and 3.0% w/v) of Na-alginate were tested in 15 g L^{-1} of Calcium chloride for complexation. Also different concentrations of Calcium chloride (5, 10, 15 g L^{-1}) were tested with 2% Na-alginate. Somatic embryos were mixed with the alginate solution (Fig.43 A&B) containing MS medium with 30 g L^{-1} sucrose and dropped into Calcium chloride for 20 min (Fig. 43 C), after that beads were washed three times using autoclaved distilled water. After hardening, the characteristics of ideal beads were evaluated. The artificial seeds were placed in pots (each pot contains five seed with five pots for each treatment) containing a MS basal medium devoid of growth regulators with 30 g/ L^{-1} sucrose and 7 g/ L^{-1} agar for one month (this experiment repeated twice) and observations, such as seed survival, were recorded.

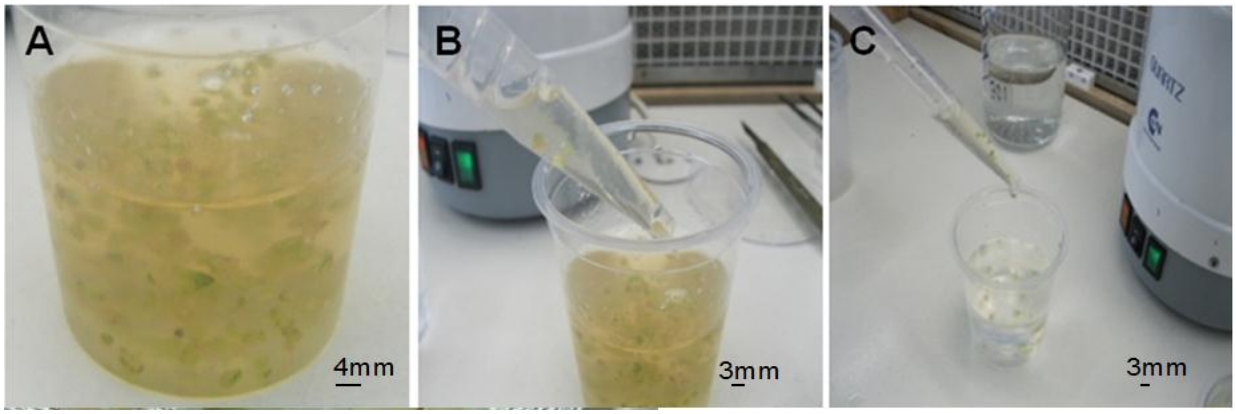


Figure 43. A) Somatic embryos mixed with Na-alginate. B) Somatic embryos with Na-alginate were pipetted using modified pipette. C) Calcium alginate beads during hardening in Calcium chloride.

4.3.1.3 Results

The use of a tyndallisation procedure for sterilization of Na-alginate was more efficient than using anautoclave as the latter reduced his gelling ability. It was observed that different concentrations of Na-alginate affect the shape and texture of beads formed in Calcium chloride. The encapsulated beads differed morphologically with different concentrations of Na-alginate and Calcium chloride. It was found that the SEs encapsulated with Na-alginate at 2 % and hardened with 15 g L^{-1} Calcium chloride were the most suitable for the production of ideal beads which were clear, isodiametric, uniform sized capsules, firm and hard enough to facilitate transfer to the culture medium and seemed to have the ability to protect the encapsulated somatic embryos (ESEs) (Fig.44) . Also the highest percentage (76%) of artificial seed survival and ease of breaking through the alginate was achieved when the beads formed in 2% Na-alginate exposed to 15 g L^{-1} of Calcium chloride after one month of *in vitro* culture on basal MS medium (Fig.45) . Although there were no significant differences between sodium alginate concentrations on survival rate of ESEs ($P = 0.294$), the beads formed using 3% Na-alginate solution were judged to be too solid and hard.

Based on the results obtained, the complexing agent calcium chloride at 15 g L^{-1} produced defined shape, clear, isodiametric, uniform beads when encapsulated with Na-alginate at 2% and produced the highest percentage of survival. However, at a low concentration of Calcium chloride (5 g L^{-1}), the beads were fragile, not firm and too soft to handle.

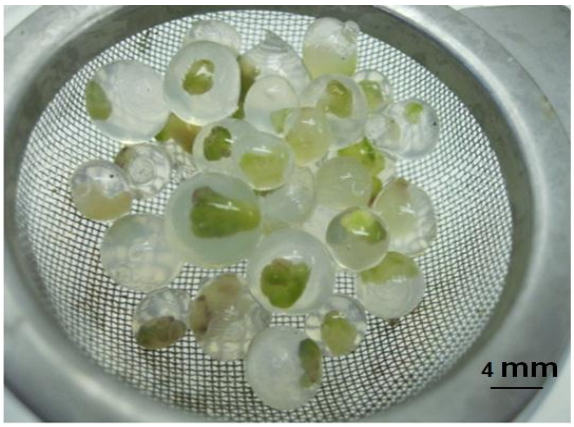


Figure 44. Encapsulated somatic embryos using 2% Na-alginate and 15 g L^{-1} calcium chloride.

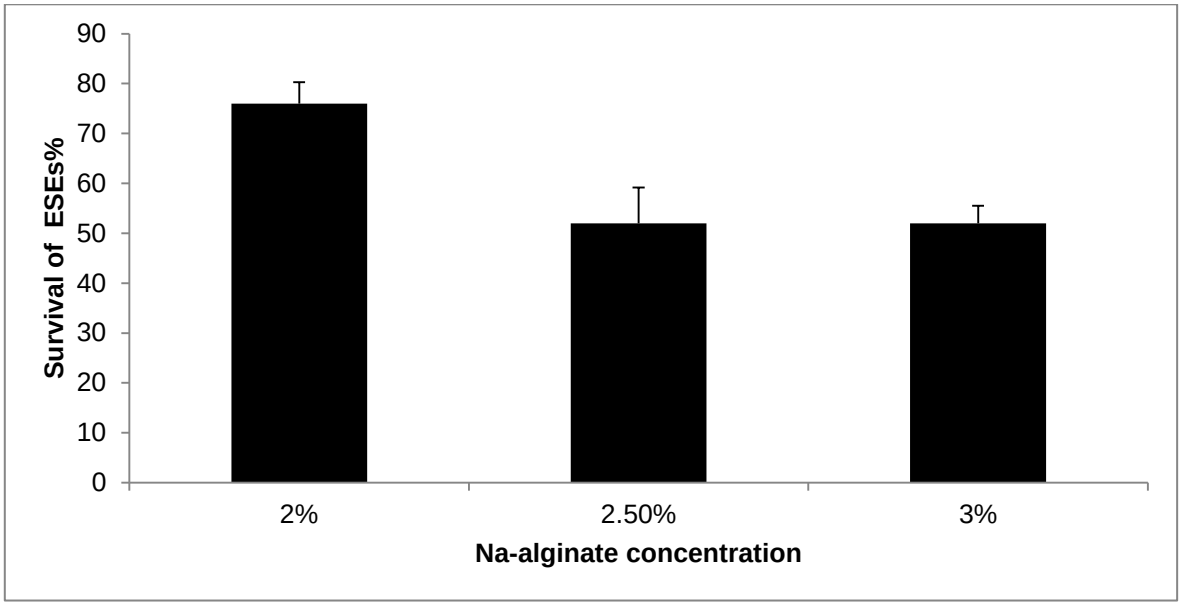


Figure 45. Effect of Na-alginate concentration on survival rate of encapsulated somatic embryos after one month of *in vitro* culture on MS medium free of growth regulators (LSD = 36.66).

4.3.2. Effect exogenous hormones added in a matrix of artificial seeds

4.3.2.1 Objective

This experiment was conducted to investigate the best hormone concentration in the artificial matrix to support germination and growth of somatic embryos.

4.3.2.2 Materials and methods

Various concentrations of Kinetin (0, 0.5, 1 mg L^{-1}) and IBA (0, 0.5, 1 mg L^{-1}) were mixed with the artificial seed matrix due to deficiency of endogenous hormones in the bead of the artificial seed. After bead formation, the artificial seeds were planted on petri dishes containing basal MS medium free of growth regulators with 30 g L^{-1} sucrose and incubated at 22.5℃ in a random distribution within an incubator for one month. Five artificial seeds were used in each petri dish and each treatment was repeated five times. The development of plantlets from artificial seed was observed and their fresh weight measured.

4.3.2.3 Results

Addition of plant growth regulators to the encapsulation solutions resulted in an increase in viability of ESEs. On the other hand, an inability of germination was observed for all concentrations of hormones used. Therefore, viability was assessed using fresh weight of SEs after one month from *in vitro* culture. Results showed that the best activity of encapsulated SEs was when Kinetin at 1 mg L^{-1} and IBA at 0.5 mg L^{-1} were used , giving the highest fresh weight (0.220 g / somatic embryo) compared with other combinations of exogenous hormone used ($P < 0.001$). The lowest fresh weight of ESEs was observed with artificial matrix devoid of growth regulators (Fig. 46) and there was no germination achieved in the absence of hormones. Other exogenous hormone concentration were mixed with the media

culture instead of matrix in the next experiment to investigate *in vitro* germination of encapsulated SEs.

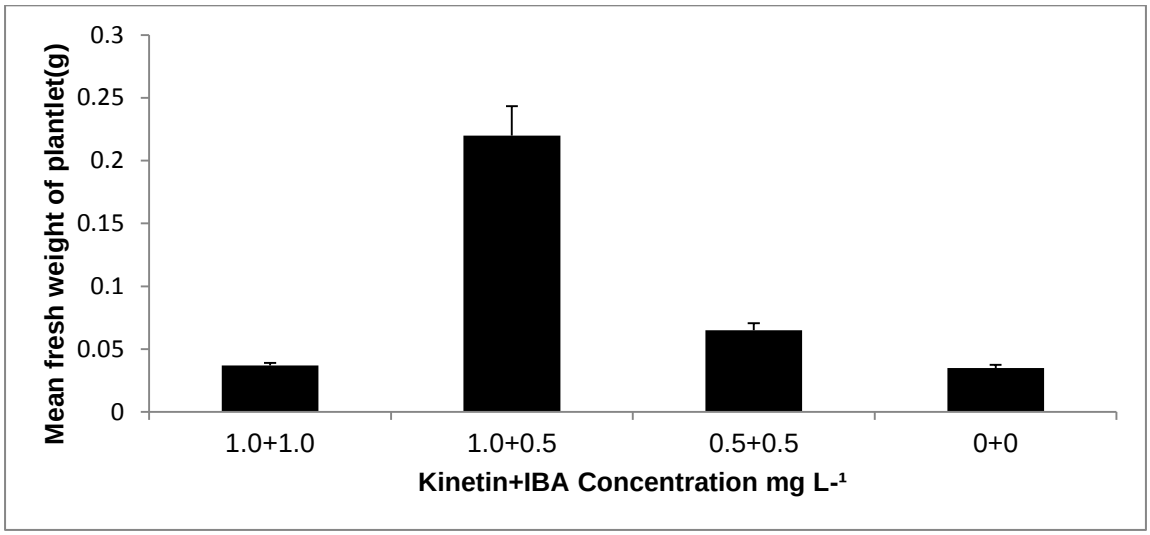


Figure 46. Effect of exogenous Kinetin and IBA added to the artificial seed matrix on fresh weight of platelets produced after one month of *in vitro* culture (LSD = 0.081).

4.3.3 Effect exogenous hormones added in culture media on germination of artificial seeds

4.3.3.1 Objective

To determine the germination ability of encapsulated somatic embryos of cauliflower on media containing various combinations of plant growth regulators.

4.3.3.2 Materials and method

Somatic embryos (3- 4mm) were encapsulated using 2% Na-alginate and 1% $Cacl_2$ with hormones Kin at 1mg L^{-1} and IBA at 0.5 mg L^{-1} and MS plus sucrose at 3% added to the matrix. Encapsulated SEs were cultured on germination media which consist of basal MS medium free of growth regulators and MS with various concentrations of Kin (0.5, 1.0, 2.0 mg L^{-1}) and IBA (0.5, 1.0, 2.0 mg L^{-1}) . The media were supplemented with 3% sucrose and 7 g L^{-1} agar. Five ESEs were placed on each petri dish and five replicates were used for each treatment. All

cultures were arranged randomly and incubated in the culture room at 22.5℃ under a 16 h photoperiod. Cultures were kept for more than one month to observe the ability of artificial seed for germination.

4.3.3.3 Results

The results revealed that the use of basal culture medium containing a combination of Kin and IBA at 1 mg L^{-1} of each gave the highest percentage (8%) of ESE germination (Fig. 47 A). There were significant differences between this culture medium and others used (P = 0. 008) (Fig. 48). Fig. 47 B shows ESEs grown on media with Kinetin 0.5 mg L^{-1} and IBA mg L^{-1} 0.5 began callusing after 4 weeks with a high incidence (76%). This treatment differed significantly from the control which was MS free of growth regulators but is not significantly different from other combinations of hormones which also showed callusing (P = 0.107) (Fig. 48). However, shoot formation was distinguished from ESEs on all media (Fig. 47 C), the highest percentage 56% was achieved on medium supplemented with Kin and IBA at 2 mg L^{-1} of each (Fig. 49).

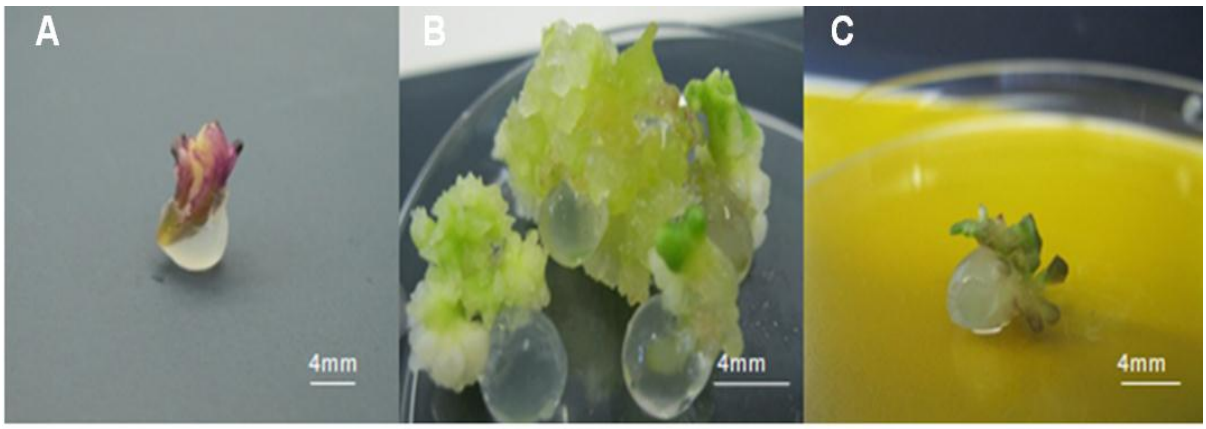


Figure 47. A) *In vitro* germination of encapsulated somatic embryo; B) Callusing from encapsulated somatic embryos on germination medium containing Kinetin 0.5 mg L^{-1} and IBA 0.5 mg L^{-1} after 4 weeks of *in vitro* culture; c) Shoot formation from encapsulated somatic embryos on germination medium containing Kinetin 2 mg L^{-1} and IBA at 2 mg L^{-1}.

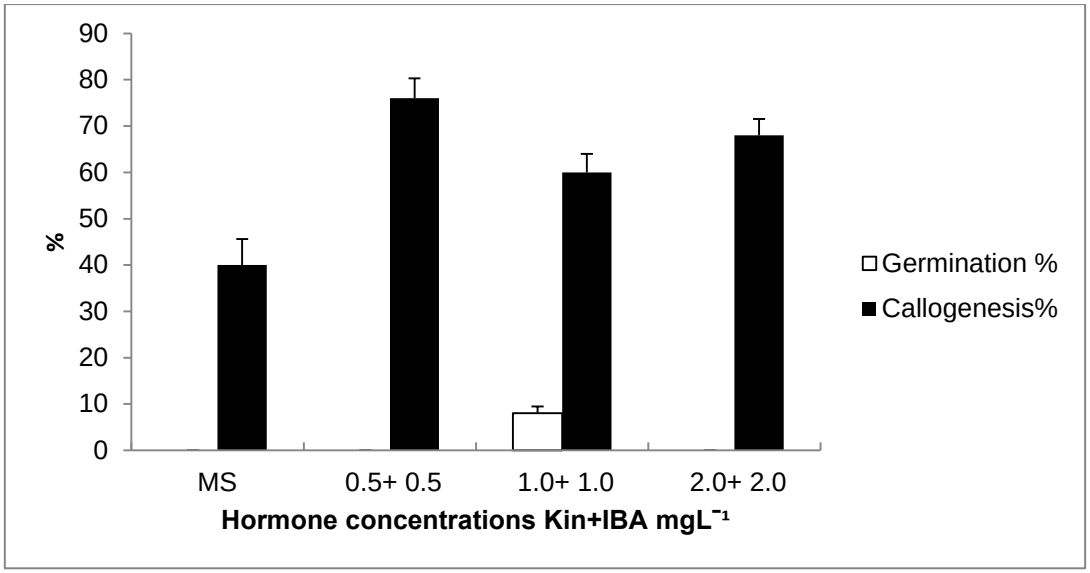


Figure 48. Effect of hormone concentration added to the culture medium for *in vitro* germination of ESEs of cauliflower after one month of culture. (LSD = 5.08 for germination and 29. 98 for callogenesis).

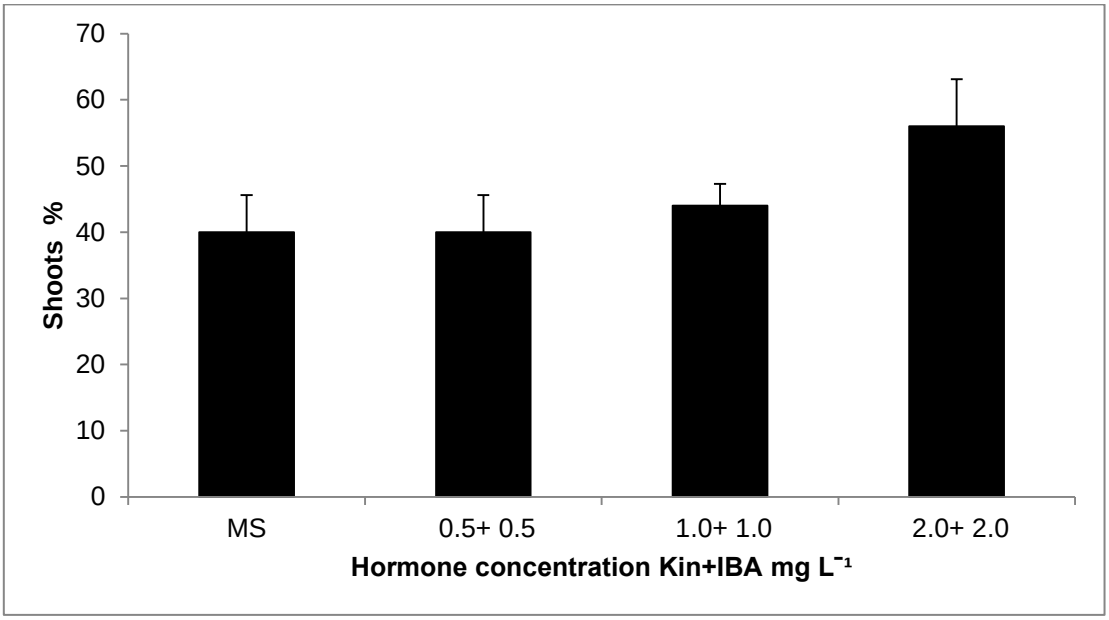


Figure 49. Effect of hormone concentration added to the culture medium for *in vitro* germination of ESEs of cauliflower on shoot formation after one month of culture (LSD = 37.68).

4.4 Disscusion

4.4.1 Effect encapsulating agents on bead formation.

An investigation of the best encapsulation matrix should consider both the physical aspects of the bead mimicking the seed coat, and the components that will support the development of the embryo into a viable seedling. This study has clearly shown that isometric, clear and firm beads encapsulating cauliflower somatic embryos can be produced. The optimum encapsulation medium with a high survival rate of SEs and subsequent easy emergence of shoots from beads was seen when somatic embryos were treated with 2% Na-alginate with the complexion timing fixed at 20 min in 15 g L^{-1} (100 mM) Calcium chloride. The same combination was reported by Rai et al., (2008) and Rihan, (2013) for encapsulation SEs of guava (*Psidium guajava* L.) and micro-shoots of cauliflower respectively to produce artificial seed. Moreover, the response in the present study presented similar findings to what was shown previously when the Na-alginate and Calcium chloride were used to produce transparent, firm and uniform artificiall seeds of protocorm-like bodies (PLB's) of orchid *Flickingeria nodosa* (Dalz.) Seidenf (Nagananda et al., 2011). Whilst a combination of 2% Na-alginate and 100 mM Calcium chloride is often used (Redenbaugh et al., 1993, Ara et al., 1999), by contrast, Tabassum et al. (2010) showed that 3% Na-alginate with 100 mM Calcium chloride was suitable for encapsulation of mature SEs of F1 cucumber (*Cucumis sativus* cv,Royal). However, here it was found that the use of 3% Na-alginate produced harder beads and this might be due to the number of Na^+ ions exchanged with Ca^+ ions as this is thought to determine the hardness or rigidity of the artificial beads (Sarmah et al., 2010). On the other hand, an increase in Na-alginate concentration could decrease respiration and germination rates (Kersulec et al., 1993), and any oxygen deficiency in the gel

bead of encapsulated somatic embryos and rapid drying might cause a decrease in conversion rate of encapsulated SEs into plants (Swamy et al., 2009). Sarmah et al. (2010) studying encapsulated PLBs of *Vanda coerulea* Grifft.ex.Lindl., have also mentioned that hardness in capsules can cause an anaerobic environment which would inhibit respiration. Thus, the concentration of the solutions used will affect the texture of the artificial seeds produced. The observations here showed that the use of a lower concentration of Calcium chloride prolonged the complexation time that is a prerequisite for ion exchange to form firm beads and to avoid over rigidity. Na-alginate is known to be of moderate viscosity and a quick gelatin in calcium chloride with low toxicity, thus it was most suitable for encapsulation (Redenbaugh, 1993). However it was noticed that the exposure of Na-alginate to high temperatures during autoclaving can cause a reduction in its gelling ability an observation also pointed out by (Larkin et al., 1988, Pattnaik et al., 1995). Since the gel production from Na-alginate does not require undue heat to sterilize it (Redenbaugh et al., 1986) a tyndallisation procedure was applied effective. This procedure was also used successfully to produce artificial seed using micro-shoots derived from cauliflower curd meristems (Rihan, 2013).

4.4.2 Effect exogenous hormones used in a matrix of artificial seed.

The components of an artificial seed that will support the development of an embryo into a viable seedling are also important and the artificial endosperm needs to be optimized in order to provide SEs with all elements necessary for growth. In the current study, the matrix of artificial seed included growth regulators and led the high viability (0.220 g/somatic embryo) of artificial seed was achieved. Despite using growth regulators in the artificial matrix, no germination was observed at any PGR concentrations used. Thus, it was necessary to add further hormones to the culture

media to enhance germination of artificial seed and this will be discussed in the next section (4.4.3). Similarly, Ma et al., (2011) stated that there was no significant effect of providing the artificial endosperm used with *Pseudostellaria heterophylla* with additives (nutrients and growth regulators) on the efficiency of germination. In another study however, a 45% germination rate was achieved from encapsulated somatic embryos of raul-beech (*Nothofagus alpine* (Poepp. &Endl.) Oerst.) when hormones were added to the artificial endoperm (Cartes et al., (2009). Nutrients should also be added to the artificial endosperm in order to maintain germplasm survival (Antonietta et al., 1998), to obtain faster explant growth (Redenbaugh et al., 1993) and to supply the energy required for germination which is normally provided by endosperm or gametophyte tissue in true seed (Ahuja, 1993). Although growth regulator combinations in artificial endosperm supported the growth of encapsulated SEs of cauliflower, further research needs to be applied to evaluate the effect of additives in the artificial matrix. For example, other concentrations or types of growth regulators and varying levels of MS (¼, ½, ¾) as well as other sucrose concentrations (1%, 2% and 4%) to assess their effect on germination of the artificial seeds. More studies are also needed to improve the ability of artificial seed to germinate and produce plantlets which can develop into plants resembling those from true seed.

4.4.3 Effect exogenous hormones used in culture media.

This study showed that the concentrations of plant growth regulators used with culture media had a positive effect on artificial seed germination. The use of a medium supplied with a combination of cytokinin and auxin resulted in the highest germination rate 8% but this rate is still low. The germination of ESEs on medium supplemented with cytokinin and auxin was reported previously in Oak artificial

seeds (Prewein and Wilhelm, 2003), *Hyoscyamus muticus* L. (Pandey and Chand, 2005) and *Catharanthus roseus* (L.) G. Don. (Maqsood et al., 2012). The results here contrast with those reported by Shigeta et al., (1993) who referred that a high germination frequency more than 95% was obtained from encapsulated somatic embryos of carrot after storage for three months when grown on polyester fiber supplied with MS medium free from hormones. Also, the germination of ESEs on MS medium free of hormone was repoted for SEs of potato (*Solanum tuberosum* L.) (Majd et al., 2010) and for SEs of vine rootstock (*V. vinifera* L. cvs. 'Chasselas' x V.*berlandieri*) (Tangolar and Büyükalaca, 2008). Moreover, Maximum conversion frequency of 55.5% was observed from encapsulated embryos of rapeseed (Brassica napus cv. Tallayeh) that cultured on MS medium free of hormones for 10 days at 4 ° C (Zeynali et al., 2013). However, low germination and conversion rates were reported with different woody species mainly due to deficiencies and asynchronous maturation of the embryonic pole, which led to difficulties in the final stages of the process (Tapia et al., 1999, Castellanos et al., 2004) cited in (Cartes et al., 2009).

Moreover, some authors consider that the degree of vigour or maturity of the embryos at the moment of being encapsulated can influence the germination of ESEs (Gomez, 1998, Nieves et al., 2001) cited in (Cartes et al., 2009). Also, previously it was suggested that encapsulation can affect embryo respiration (Redenbaugh, 1990) and this in turn might influence the germination and viability of somatic embryos (Nair and Gupta, 2007).

There is a risk involved in the use of a combination of cytokinin and auxin in the germination mediu in that it can increase callus induction (Harish et al., 2010). In the current investigation it was noticed that the addition of cytokinin and auxin in balance

in culture media led to callus formation from ESEs. The morphogenic response might be controlled by the hormonal balance represented by the ratio of cytokinin to auxin (Al-Ramamneh, 2006). Similarly, Ahmad and Spoor (1999) as well as Mungole et al., (2009) referred to a high callus production from explants in curly kale (*Brassica oleracea* L.*)* and *Ipomoea obscura* L. respectively when the same concentration of both cytokinins and auxin were used (see chapter two).

It has been observed that encapsulated SEs formed multiple shoots on all media used for germination. This might be due to the presence of high levels of cytokinin which exist in the artificial matrix and when supplemented by the culture medium it enhanced cell division and shoot formation. These results are supported by Pandey and Chand, (2005) who reported that encapsulated SEs of *Hyoscyamus muticus* L. exhibited shoot induction when cultured on MS media supplemented with cytokinin and auxin. Similarly, the highest number of shoots was obtained from encapsulated bulblets of garlic (*Allium sativum* L.) when using medium supplemented with 2 mg L^{-1} BA and 2 mg L^{-1} NAA (Bekheet, 2006).

4.5 Conclusion

It is important to produce artificial seeds similar to true seeds with sexual embryos. This study was conducted to investigate the best artificial seed matrix that can maintain the viability of SEs. The encapsulation of SEs produced from RDECT was optimal when 2% of Na-alginate was polymerized in 15 g L^{-1} (100 mM) Calcium chloride. The *in vitro* encapsulated somatic embryos showed prolonged viability and retained a capability to germinate into plantlets and demonstrated that they can provide an alternative method for micropropagation of cauliflower plants and the system has the potential to be applied when plants have a problem with seed production e.g. when maintaining inbred lines. The development of artificial seed

from SEs can be considered a good tool for mass propagation of cauliflower but requires further optimisation before commercialisation.

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Chapter Five

Cryopreservation of embryogenic callus tissue and somatic embryos

5.1 Introduction

5.1.1 Cryopreservation of embryogenic tissues

Cryopreservation of embryogenic tissue is an important storage step in seedling production via somatic embryogenesis and in genotype selection (Kong and Aderkas, 2011). Ideally long–term storage of selected plant material can be achieved by cryopreservation at the ultra-low temperature of liquid nitrogen (LN, -196ºC) (Haggman et al., 1998, Lelu-Walter et al., 2006). Embryogenic cells tend to be highly cytoplasmic and contain less water for lethal ice formation and thus can be considered ideal materials for cryopreservation (Finer, 1994). In many laboratories across the world, the use of somatic embryogenesis is being increasingly applied *in-vitro* for plant breeding as it can provide a high efficiency of proliferation and brings with it the attraction of cryopreservation of germplasm stocks (Misson et al., 2006, Hargreaves and Menzies, 2007). Many different types of plant material can be cryopreserved in this method, such as, embryonic axes isolated from seeds, vegetative propagules including pollen, apical or axillary buds, somatic embryos and embryonic callus tissues (Engelmann, 2004). Cryopreservation of embryogenic tissues can be considered as an essential storage step in seedling proliferation and genotype selection through somatic embryogenesis (Kong and Aderkas, 2011) which can differentiate to form somatic embryos at a later time (Namasivayam, 2007). The risk of loss of embryogenicity of embryongenic tissues (partially or entirely) can be increased by long-term passaging and sub-culturing and there is an ever increasing risk of somaclonal variation through the sub-culture of actively proliferating tissues. Undesirable changes that take place during successive subcultures *in vitro* may be prevented through the cryostorage of embryonic tissues (Malabadi and Nataraja, 2006) since the ultra-low temperature stop cellular metabolic functions (Kartha,

1981). By this method maximal stability of phenotypic and genotypic behavior of stored germplasm can be achieved as well as providing minimal storage space and minimal maintenance requirements (Suzuki et al., 2008). Cryopreservation enables a limit to the number of subcultures and also reduces the risk of microbial contamination in the stored cultures (Malabadi and Nataraja, 2006). As an alternative to traditional clonal storage at growing temperatures with frequent sub-culturing, some embryogenic materials can be stored as tissue cultures at normal refrigeration temperatures (Westcott et al., 1977). This method of storing germplasm is referred to as cold storage of cultures and involves using incubators running at 0 to 15°C. In this way, fewer transfers are required to limit culture growth (Aitken-Christie and Singh, 1986) and it can be considered a convenient method to preserve germplasim (Westcott, 1981). The successful storage system requires properties such as 1) The ability to reduce the growth and development of *in vitro* plants to provide intervals between subculture and other handling in order to achieve a positive extended sub-culture timespan 2) Maintenance ability with retention of the highest possible level of viability of the stored material with minimized risk to genetic stability. 3) The ability of stored material to retain the full development and functional potential when it is returned to the physiological temperatures. 4) The capability to achieve a significant reduction in cost of labour input, materials and commitments of specialized growing facilities (Grout, 1995). Thus*, In vitro* storage based on slow growth techniques is pointed out as alternative strategies can be applied for conservation of genetic resources of plants (Kaviani, 2011).

5.1.2 Cryopreservation techniques

Many factors can affect freezing tolerance in the freezing protocol such as cryoprotection agents, pre-treatments, freezing and thawing procedures, and post-thawing treatments (Vicient and Martínez, 1998). Cryopreservation can be achieved through quick-freezing and storage in L.N or by gradual lowering of temperature 1℃/min to -40 ℃ followed by immersion and storage in L.N for the desired period (Jain et al., 2000b). During slow cooling, increases in the concentration of intracellular solutes can be achieved as the intracellular water moves out and is frozen extracellularly (Efendi, 2003). After cryostorage, thawing needs to be applied to the plant material and this must not threaten viability and so needs to be optimised (Jain et al., 2000). The correct post-thaw treatment of cryopreserved cells is essential to achieve survival and re-growth of the plant material (Lynch et al., 1994). Normally the viabilitry and regeneration potential of ex-cryopreserved material is tested in *in-vitro* culture (Jain et al., 2000). The high water content of living plant cells normally makes the partial dehydration of plant tissue a prerequisite for successful cryostorage by preventing freezing injury (Matsumoto et al., 1994) caused by intracellular ice crystal formation (Sakai, 1960). The removal of water can be achieved by direct dehydration or using chemical (osmotic) dehydration (Matsumoto et al., 1994). Successful cryopreservation requires avoiding ice crystal formation inside cells during both freezing and thawing. This essential requirement can be achieved using different pretreatmens such as cold acclimation, immersion in concentrated sugar solutions, exposure to ABA or extensive dehydration in air (Shibli et al., 1998, Shibli, 2000, Ashmore, 1997). Previous research has revealed that sucrose and glucose can be used to induce desiccation tolerance effectively by means of osmotic dehydration (Suzuki et al., 1998). The successive osmotic and

evaporative dehydration of plant cells is frequently a basis for successful cryopreservation and is dependent on encapsulation-dehydration techniques (Swan et al., 1999). Also compared to other methods, using the encapsulation-dehydration technique can avoid the use of a harmful cryoprotectants (Shibli et al., 1998, Moges et al., 2004). DMSO is frequently used in cryopreservation protocols but can cause toxicity which is a major problem in vitrification techniques, while the use of non-toxic materials such as sucrose can be applied by encapsulation – dehydration techniques (Lipavska and Vreugdenhil, 1996, Ashmore, 1997). A preculture-dehydration technique (Dumet et al., 1993d) was used in the current study to preserve cauliflower root-derived embryogenic callus tissue (RDECT) and an encapsulation – dehydration technique for SEs cryopreservation. To the author's knowledge, to date, there are no reports in the literature on the cryopreservation of either cauliflower RDECT or SEs. This study therefore aimed to investigate the prospects of cryopreservation of embryogenic callus tissue and somatic embryos of cauliflower.

5.2 Experiments

5.2.1 Long term storage of ECT by cryopreservation in liquid nitrogen using preculture-dehydration technique

5.2.1.1 Effect of preculture treatment with various concentration of sucrose and duration of preculture on survival of RDECT.

5.2.1.1.1 Objective

To investigate the effect of preculture duration and sucrose concentration on viability of RDECT.

5.2.1.1.2 Materials and methods

RDECT clusters (one year old) were weighed (Oxford- Model A 2204 balance; 1.5 g for each replicate). ECT clusters were placed on 9 cm sterile petri dishes containing 20 mL semi solid MS medium supplemented with increasing concentrations of sucrose (0.25, 0.50, 0.75 and 1.0 M) (preculture media) as well as a control treatment of 0.1 M (which was used throughout as basal medium) for two periods (24h and 7 days). Cultures were incubated at 22.5 ^{o}C under 16h photoperiod using cool, white fluorescent light. Following preculture treatments, the ECT were harvested and cultivated on petri dishes containing callus proliferation medium, the same CIM containing 0.5 mg L^{-1} 2, 4-D and 1 mg L^{-1} Kinetin and incubated for 14 days. Three replicates were used for each treatment (two petri dishes for each replicate). The survival of ECT was assessed as an average net weight (increase or decrease in fresh weight) as follows:

Net weight of RDECT = T2-T1

T2 = Fresh weight of precultured RDECT after 14 days of in vitro culture.

T1 = Fresh weight of precultured RDECT after preculture period.

5.2.1.1.3 Results

The survival of RDECT increased as the preculture duration increased from 24h to 7 days ($P = 0.049$). The results showed that there was no significant effect for sucrose concentration on growth of RDET compared to the Control. The highest mean of net weight (1.063 g) was achieved from preculture on 0.75M sucrose. This concentration was applied in all subsequent experiments (Fig. 50).

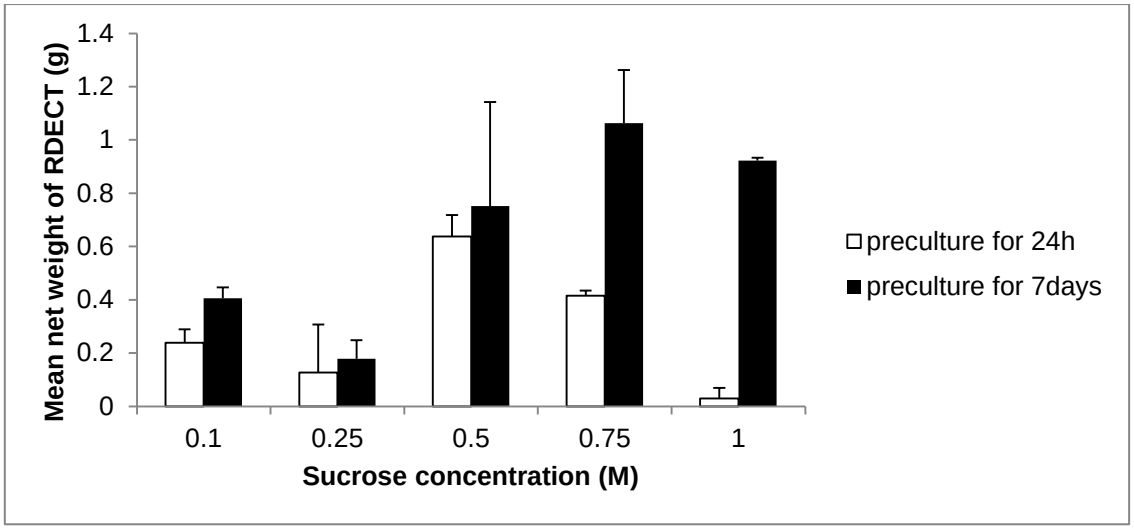

Figure 50. Effect of preculture treatments for 24h and 7 days at varying sucrose concentrations on mean net weight of RDECT of cauliflower after 14 days of *in vitro* culture (LSD = 0.8).

5.2.1.2 Effect of dehydration treatments on survival of ECT

5.2.1.2.1 Objective

To investigate the effect of dehydration duration on the water content and viability of RDECT.

5.2.1.2.2 Materials and methods

Precultured RDECT clusters were grown on medium with 0.75 M sucrose for 7 days after which clusters were used to determine the appropriate time for dehydration. RDECT clusters were placed on a piece of pre-weighed aluminum foil in an uncovered petri dish and dehydrated under a sterile air flow in a laminar flow cabinet for 8 time periods (0, 30, 60, 90, 120, 150, 180 and 210 min). The weight of the RDECT clusters with aluminum foil were taken at the end of each dehydration treatment, then samples (three replicates for each treatment) were wrapped in aluminium foil and dried in an oven set at 80 ºC for 96h using the low constant temperature oven method (ISTA, 2005) to determine moisture content (MC) which was evaluated as follows:

MC%= [(W2-W3) / (W2-W1)] *100

Where W1 = weight of aluminum foil, W2 = weight of aluminum foil + ECT before drying, W3 = weight of aluminum foil + ECT after drying.

Dehydrated clusters were then cultivated on semi solid callus MS proliferation medium (CIM) containing 0.5 mg L^{-1} 2, 4-D and 1 mg L^{-1} Kinetin and cultured at 22.5 ºC for 14 days. Cultures (three replicates of each treatment) were weighed after 14 days culture to assess the effect of dehydration treatments on subsequent growth of the RDECT. These two experiments, determination of water content and callus growth after dehydration, were repeated twice.

5.2.1.2.3 Results

As expected the dehydration treatments caused a significant decrease in fresh weight of precultured RDECT (P = 0. 001). It was noted that air-drying for a 30, 60 and 90 min in a laminar flow bench for RDECT resulted in a non-significant decrease in fresh weight after 14 days of subsequent culture compared to the Control (without dehydration). The mean fresh weight for these treatments was 0.802 g, 0.734 g and 0.711 g respectively (Fig. 51). Longer drying times of 120 and 150 min significantly reduced mean fresh weight to 0.355 and 0.334 g respectively and the lowest weight of RDECT was achieved when 180 and 210 min dehydration period were applied. The dehydration process led to a decrease in the moisture content of RDECT from 73.87 % to 62.50 % (Fig. 52). Dehydration of RDECT for 90 min significantly reduced the moisture content to 68.50% and the growth of dehydrated callus tissue which assessed as mean fresh weight after 14 days of *in vitro* culture does not differ significantly from the control. Thus, 90 min can be used

as a partial drying-period of the precultured RDECT in subsequent cryopreservation experiments.

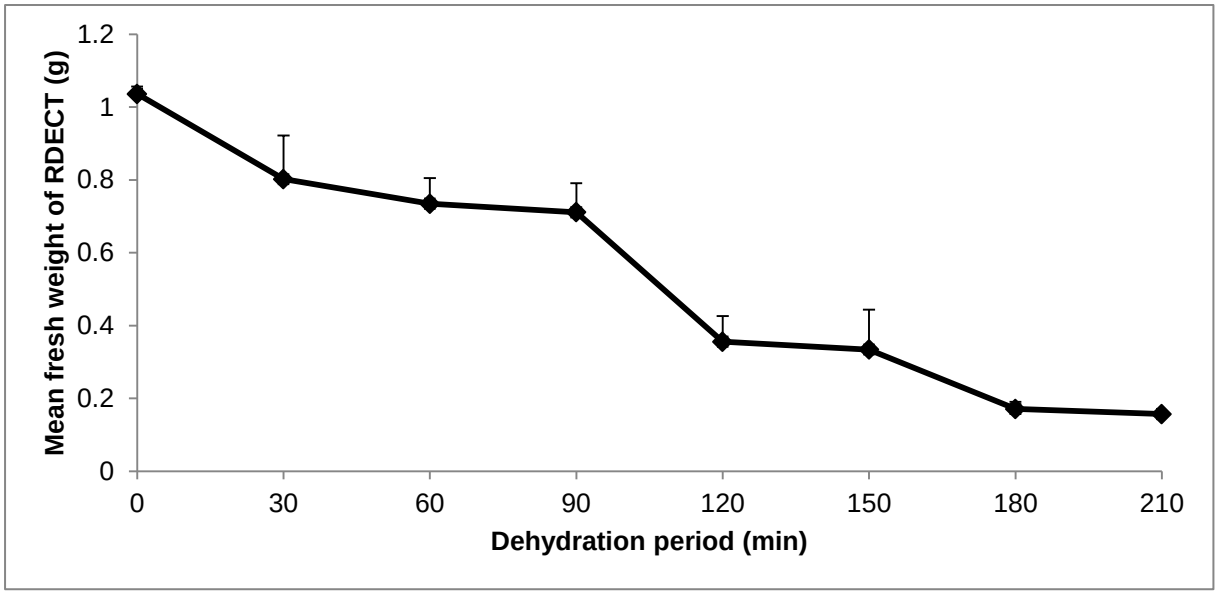

Figure 51. Effect of dehydration periods on subsequent growth of precultured RDECT, assessed as fresh weight after 14 days of *in vitro* culture on CIM (LSD = 0.39).

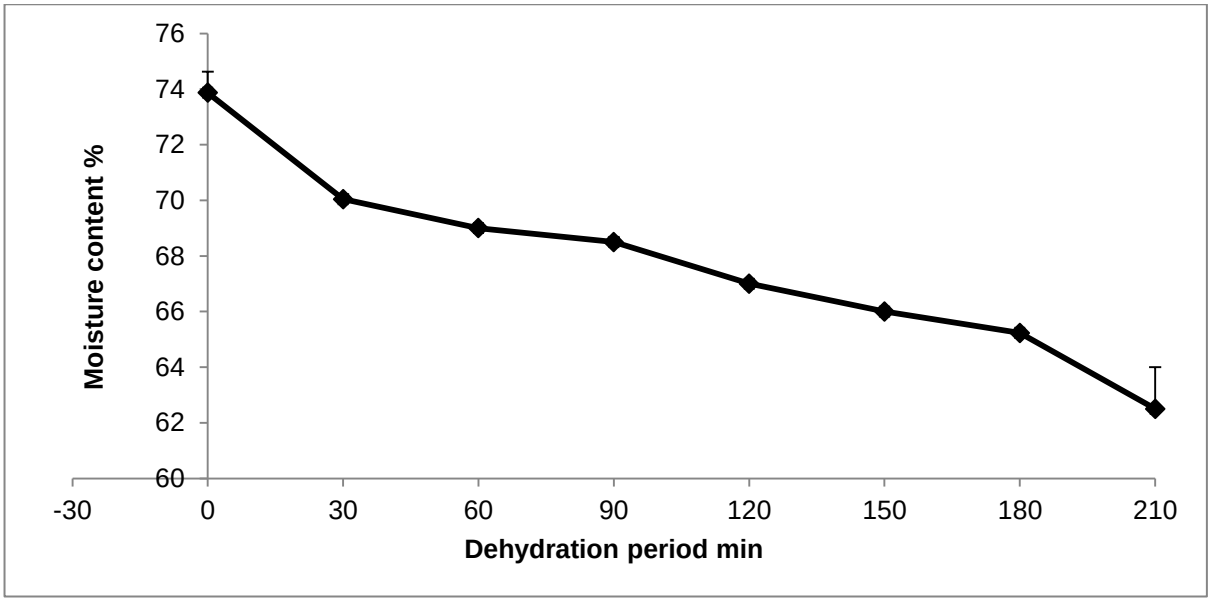

Figure 52. Effect of dehydration periods of cauliflower RDECT on moisture content of precultured RDECT (LSD = 3.14).

5.2.1.3 The effect of different dehydration-cryopreservation protocols on embryogenecity of RDECT.

5.2.1.3.1 Objective

To evaluate different dehydration-cryopreservation protocols and to test the best protocol that can lead to form somatic embryos from cropreserved RDECT.

5.2.1.3 .2 Materials and methods

Cryopreservation protocols

Five 1.5 g clusters of RDECT were precultured on petri dishes containing semi solid basal MS medium enriched with 0.75 M sucrose for 7 days. These precultured RDECT clusters were then dehydrated on uncovered sterilized pteri dishes for 90 min. The semi-dehydrated RDECT clusters were then transferred into 2 mL sterile cryovials. The vials were tightly closed and then either frozen directly in LN for 1h in a Dewar flask (Fig. 53 A) or by a slow cooling method using a programmable freezer (KRYO 10 series cryo-freezer) (Fig. 53 B). The slow cooling programme was 1 °C/min until -20 or -40 ^{o}C as a terminal temperature. As a control fresh RDECT clusters were immersed directly in LN. The protocols that were applied in this study are described more fully as follows:-

Protocol 1: Fresh RDECT (non precultured) + Direct immersion in LN.

Protocol 2: Preculture with 0.75 M sucrose for 7 days at 5℃ + dehydration (90 min) + direct immersion in LN for 1h

Protocol 3: Preculture with 0.75 M sucrose for 7 days at 10℃ + dehydration (90 min) + direct immersion in LN for 1h.

Protocol 4: Preculture with 0.75 M sucrose for 7 days at 5℃ + dehydration (90 min) + cryo-freezing to -20 + LN for 1h.

Protocol 5: Preculture with 0.75 M sucrose for 7 days at 10℃ +Dehydration (90 min) + cryo-freezing to -20 + LN for 1h.

Protocol 6: Preculture with 0.75 M sucrose for 7 days at the 5℃ +dehydration (90 min) + cryo-freezing to -40 + LN for 1h.

Protocol 7: Preculture with 0.75 M sucrose for 7 days at 10℃ +dehydration (90 min) + cryo-freezing to -40 + LN for 1h.

After each protocol the cultures were thawed rapidly at 40 ºC for 3 min. Vials were plunged into a water bath in a manner to prevent penetration of water inside the vials. The frozen RDECT clusters were transferred to liquid basal MS medium containing 1.2 M sucrose for 10 min. The cultures were maintained in callus induction and proliferation medium (CIM). Five replicates (three petridishes for each replicate) were used for each protocol. The survival of RDECT clusters in each petri was recorded after 21 days of subsequent *in vitro* culture.

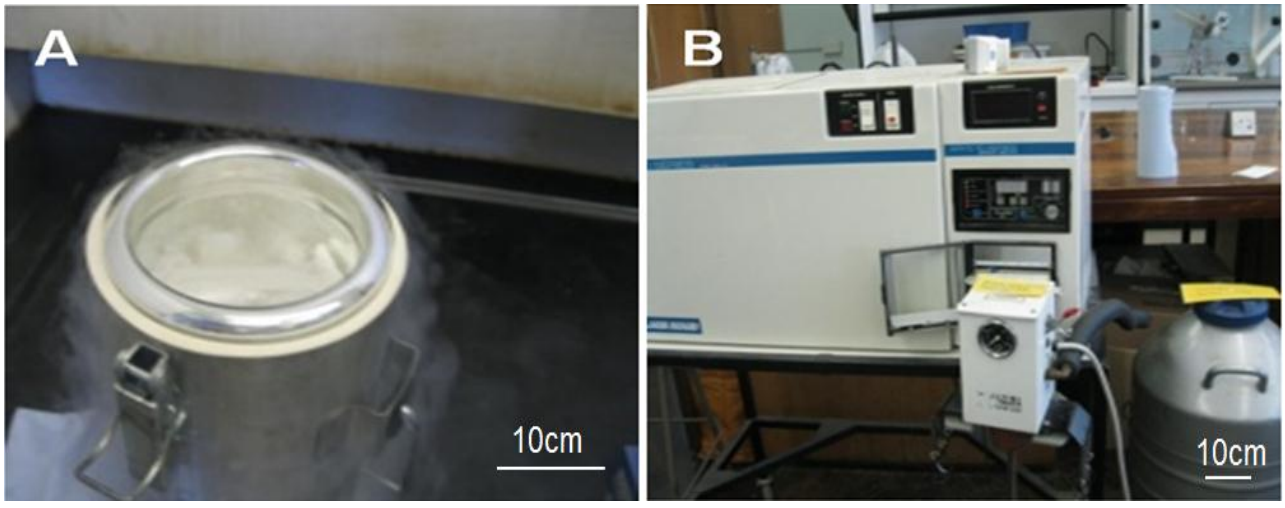

Figure 53. A) Dewar flask used for direct immersion in LN. B) Programmable freezer (KRYO 10 series cryo-freezer).

Embryogenecity assessment of cryopreserved RDECT.

After 21 days from subculture of cryopreserved RDECT, explants from the 600 µm sieve size produced using CIALM technique were placed in agitated liquid SIM (74 µL for each pot which containing 30 mL of SIM) to assess the embryogenecity of cryopreserved RDECT. Embryogenicity was defined as the capability of the tissue to form somatic embryos and assessed as the proportion of explants that subsequently formed embryos.

The number of SEs that formed on each explant after 40 days of *in vitro* culture was counted under binocular light microscope. Five replicates (three pots for each replicate) were used for each treatment.

5.2.1.3.3 Results

Cryopreservation protocols effect

It was found that all cryopreservation protocols used led to survival of the RDECT after 21 days of *in vitro* culture in CIM medium (P = 0. 008). RDECT clusters were re-initiated to grow and the proliferation of frozen RDECT increased rapidly (Fig.54). The highest survival rate was observed using protocosl 1 and 2. Most protocols tested produced high level of survival except protocol 7 which differed significantly from all other protocols with the lowest rate of survival (72%) (Fig. 55).

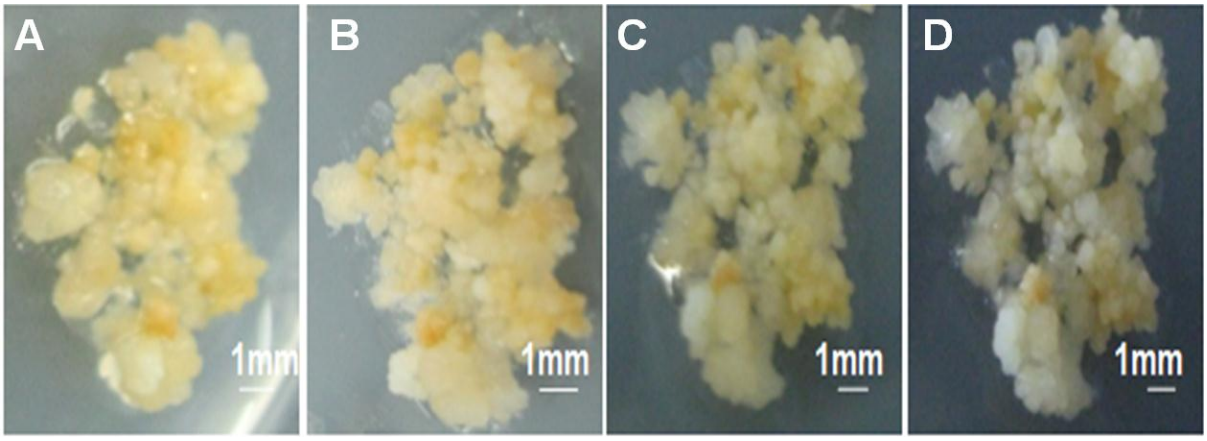

Figure 54. Survival of cryopreserved cauliflower RDECT from direct immersion in LN after A) thawing B) 7days C) 14 daysD) 21 days of *in vitro* culture on callus induction and proliferation medium (images appear here for one cluster of RDECT).

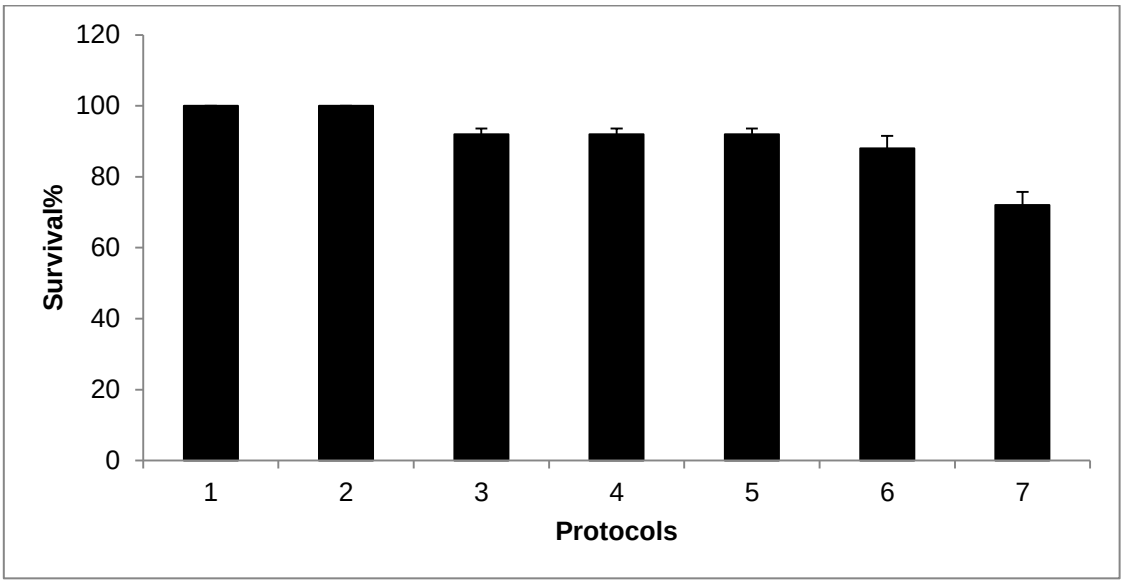

Figure 55. The effect of cryopreservation of RDECT clusters in LN for 1h on survival rate after 21 days of *in vitro* culture on CIM (LSD = 14.2).

Embryogenecity assessment

It was observed that after 20 days of culture of cryopreserved RDECT in agitated liquid SIM, somatic embryos started to appear from the explants of protocols 2 and 3 only (Fig. 56). After 40 days, protocol 2 was significantly higher than protocol 3 but all other protocols retained no embryogenic competence (Fig. 57). This experiment

was preliminary in nature and did not provide recovery values for material processed for cryopreservation but not frozen. Consequently, the positive recovery achieved with the 2 successful protocols cannot be assessed against control values and the mortality caused by different parts of the protocols cannot be assessed.

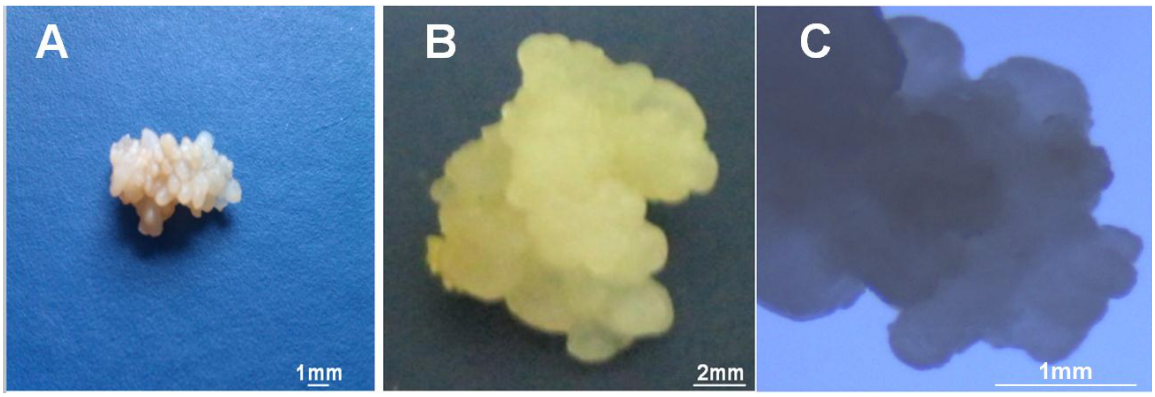

Figure 56. Somatic embryos at globular stage which developed from RDECT frozen in LN for 1h after preculture on A) 0.75 M sucrose at 10℃ for 7 days and dehydration period 90 min. B and C) 0.75 M sucrose at 5℃ for 7 days and dehydration period 90 min. The appearance of SEs was after cultivation in liquid somatic induction medium (SIM) for 40 days.

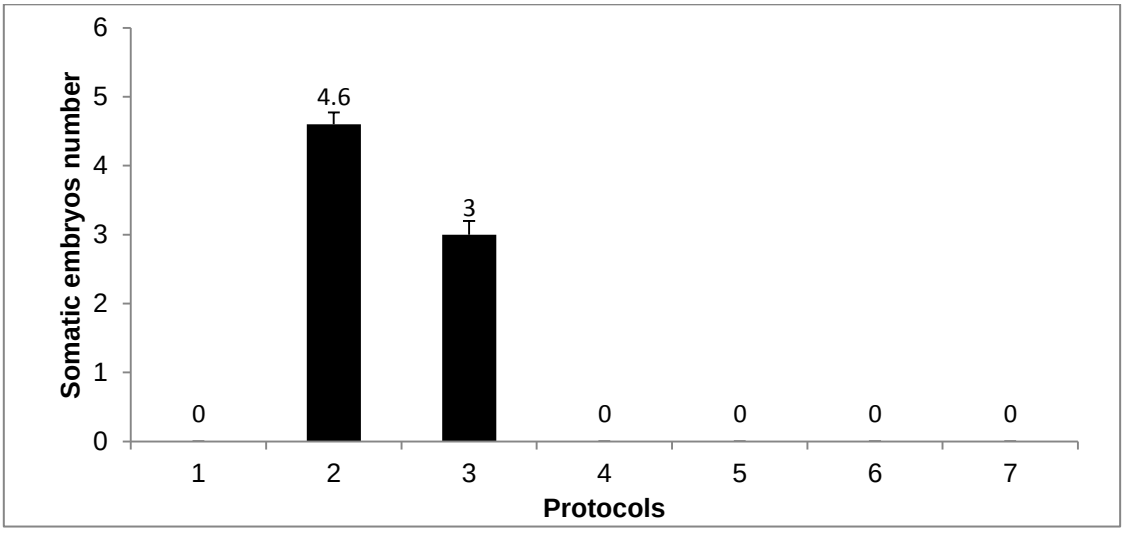

Figure 57. Effect of cryopreservation protocols on somatic embryo formation after 40 days of culture on agitated liquid SIM. (LSD = 0. 65).

5.2.2 Short term storage of ECT by cold storage at 5°C.

5.2.2.1. Objective

The investigation of the capacity of RDECT for cold storage at low non-freezing temperatures and to determine the best duration for storage.

5.2.2.2 Materials and methods

Culture materials and conditions

Pieces of RDECT two years old (5 mm in diameter) were placed in petri dishes containing CIM. Five petri dishes per replicate containing five pieces each were used. Three replicates were distributed at random in a refrigerator at 5 ℃ under darkness. After three months of cold storage, the cultures were removed and evaluated for their ability to produce somatic embryos and the number of SEs per explant recorded.

Embryogenicity assessment of stored RDECT.

The embryogenicity of stored and non stored RDECT (which had been maintained continuously on CIM by subculturing) was assessed using the CIALM technique. Pieces of RDECT were transferred to a blender in order to produce 600 µm sized explants. A constant volume of 74 µL of explants was placed in pots containing 30 mL of liquid SIM. Five replicates of each treatment were used (three pots per each replicate). Cultures were placed on a rotary shaker supplemented with 16h light (spectral photo fluency 40 µmol m^{-2} s^{-2}) supplied by cool white fluorescent tubes and cultured for 40 days. The embryogenecity and somatic embryos per explant were assessed under a binocular light microscope periodically during culture.

5.2.2.3 Results

The results showed that the viability of cold stored RDECT was maintained. When the stored RDECT was transferred to agitated liquid SIM somatic embryo development that started with the globular stage was easily detected on explants after only 20 days of culture. After that, all other SE development stages were distinguishable. The total number of somatic embryos produced after 40 days of culture did not differ significantly from the number produced in Control cultures (P = 0. 830) and embryogenicity rate (60%) was similar in stored and Control cultures. Also, It was noted that there were no differences among all developmental stages except the torpedo stage which were significantly decreased (P = 0.032). (Table. 3).

Table 3: The effect of callus tissue type on SEs formation and embryogenecity rate in RDECT under cold storage (5 oC) and non-cold storage

Callus tissue type	Total number of SEs	Number of globular SEs	Number of heart SEs	Number of torpedo SEs	Number of cotyledonary SEs	Embryogenecity%
Non stored RDECT (control)	25.2a	6.8a	5.6a	4.4a	8.4a	60a
Stored RDECT	24.4a	5.8a	5.0a	2.0b	11.6a	60a
Mean	24.8	6.3	5.3	3.2	10.0	60
LSD	8.3	2.8	1.7	2.1	4.0	6.3

5.2.3. Long term storage of somatic embryos by cryopreservation in liquid nitrogen (LN) using encapsulation-dehydration technique

5.2.3.1. Effect of preculture treatment with various concentration of sucrose and duration of preculture on survival of Encapsulated SEs.

5.2.3.1.1 Objective

To investigate the effect of preculture duration and sucrose concentration on survival of Encapsulated SEs (ESE's).

5.2.3.1.2 Materials and methods

Encapsulation of SEs.

Somatic embryos at cotyledonary stage (3-4 mm length) were gathered after 40 days from the culture on agitated liquid somatic induction medium SIM, and encapsulated (see section 4.3.3.2 for details) The beads were washed three times with sterilized distilled water and only bead containing one SE were selected for further experimentation.

Preculture treatments

In order to optimize the pre-culture period, ESEs were precultured on MS medium supplemented with various concentration of sucrose (0.1, 0.25, 0.50, 0.75 and 1.0 M) and incubated on a rotary shaker at 22.5 ^{o}C under 16 photoperiod using cool, white fluorescent light for two periods 24h and 7 days. The survival rate for ESEs was calculated after 14 days of *in vitro* culture on MS medium.

5.2.3.1.3 Results

The survival rate of ESEs after 7 d preculture differed significantly those for 24h ($P = 0.047$). The highest survival rate (80%) was obtained after preculture on MS medium supplemented with 0.75 M sucrose for 7 days (Fig. 58) but this did not differ

significantly from the control treatment (0.1 M) or from 0.5 M sucrose but differed significantly from the other sucrose concentration. Following this assessment, preculture with 0.75 M sucrose for 7 days was chosen as optimal and applied in the following experiments.

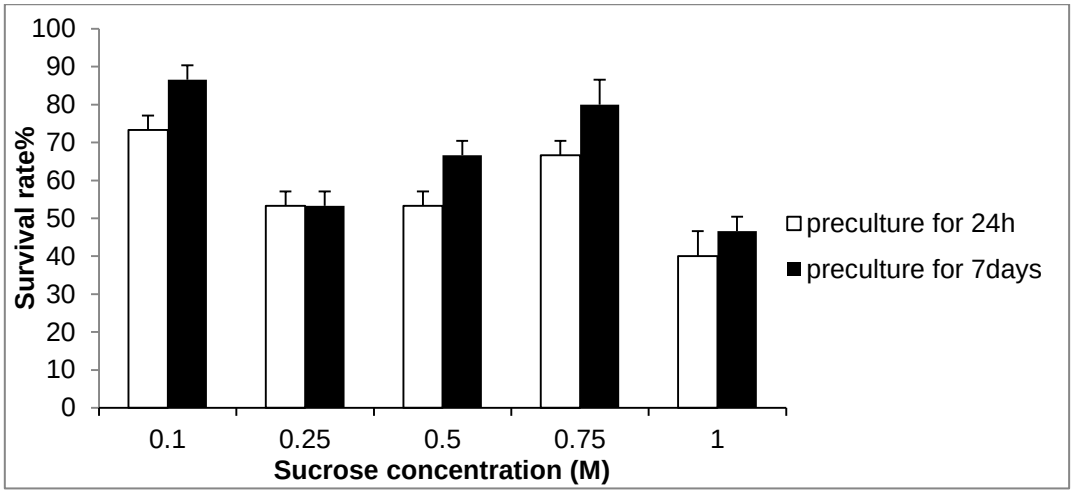

Figure 58. Effect preculture treatments for 24h and 7 days with varying sucrose concentrations on survival rate of encapsulated somatic embryos after 14 days of *in vitro* culture on semi-solid MS medium (LSD = 24. 8).

5.2.3.2 Dehydration of ESEs

5.2.3.2.1 Objective

To determine the best dehydration period that can be used to reduce the water content yet maintain the viability of ESEs after dehydration.

5.2.3.2.2 Materials and methods

Precultured beads were placed on pieces of pre-weighed aluminum foil in uncovered petri dishes and dehydrated under sterile air flow in a laminar flow cabinet for eight time periods including 0, 30, 60, 90, 120, 150, 180 and 210 min. The weight of ESEs with aluminum foil were taken after each dehydration treatment and then samples wrapped in the aluminium foil for dry weight determination and moisture content (MC%) calculated as previously (5.2.1.2.2).

Dehydrated beads were cultivated on semi-solid MS medium and were maintained at 22.5 ºC for 14 days and their growth observed. The survival rate of the ESEs was recorded after 14 days of culture.

5.2.3.2.3 Results

It was observed that the dehydration process had a significant effect on survival of ESEs after 14 days of *in vitro* culture (P = 0.003) (Fig. 59). The results showed that 120 mins dehydration was the best with the highest value (88%) for the survival of dehydrated ESEs (Fig. 60). As expected (Fig. 61) prolonging dehydration resulted in a deceasing moisture content of the ESEs from 82.21% to 73.05%. The 120 min dehydration period reduced the moisture content to 79.5%. 120 min dehydration was used in all subsequent experiments.

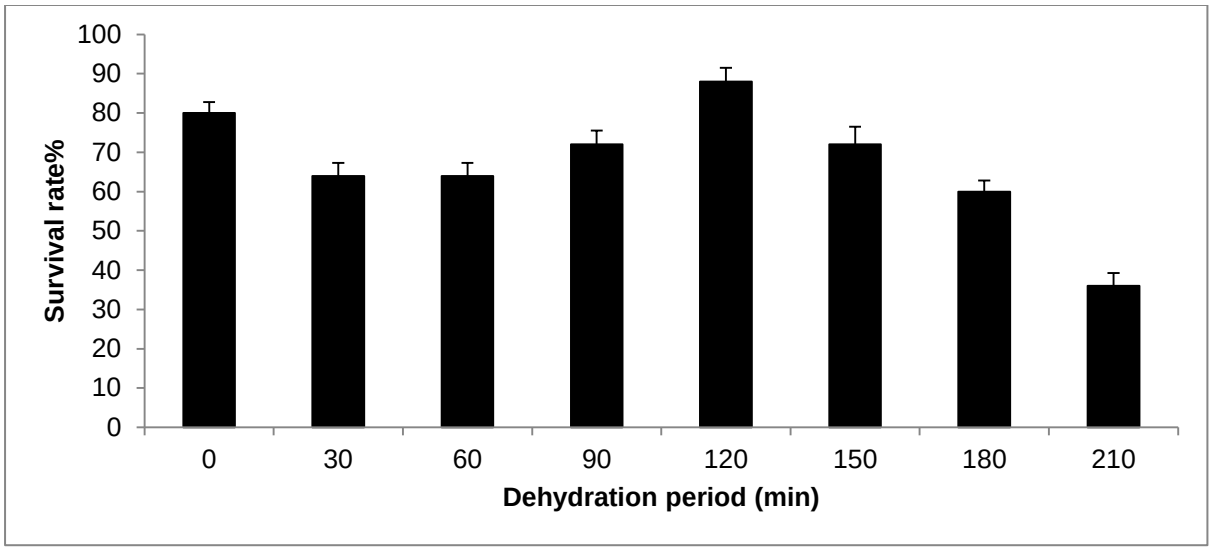

Figure 59. Effect dehydration period on survival rate of precultured encapsulated somatic embryos after 14 days of *in vitro* culture on semi-solid MS medium (LSD = 22.3).

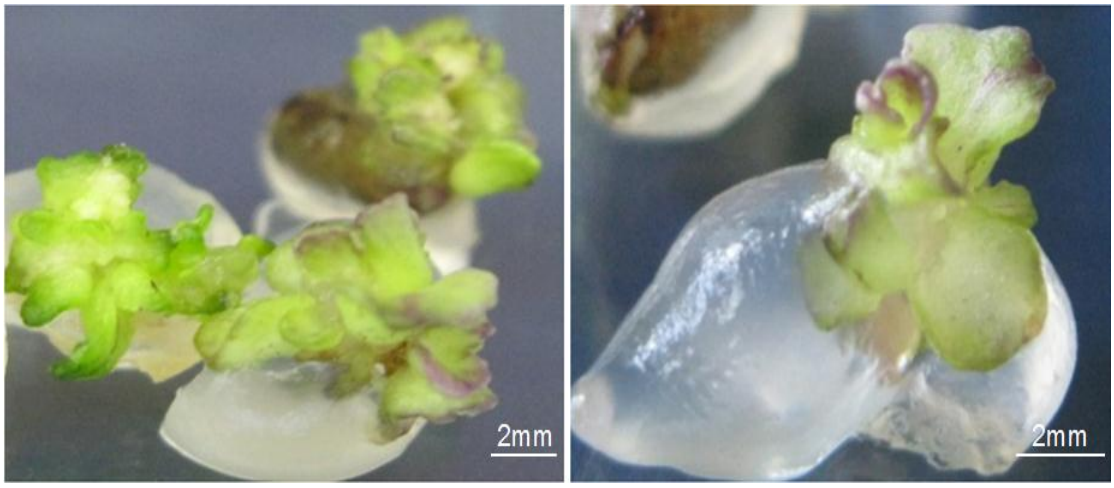

Figure 60. Survival of ESEs that dehydrated for 120 min in laminar flow and cultivated for 14 days of *in vitro* culture on semi-solid MS medium.

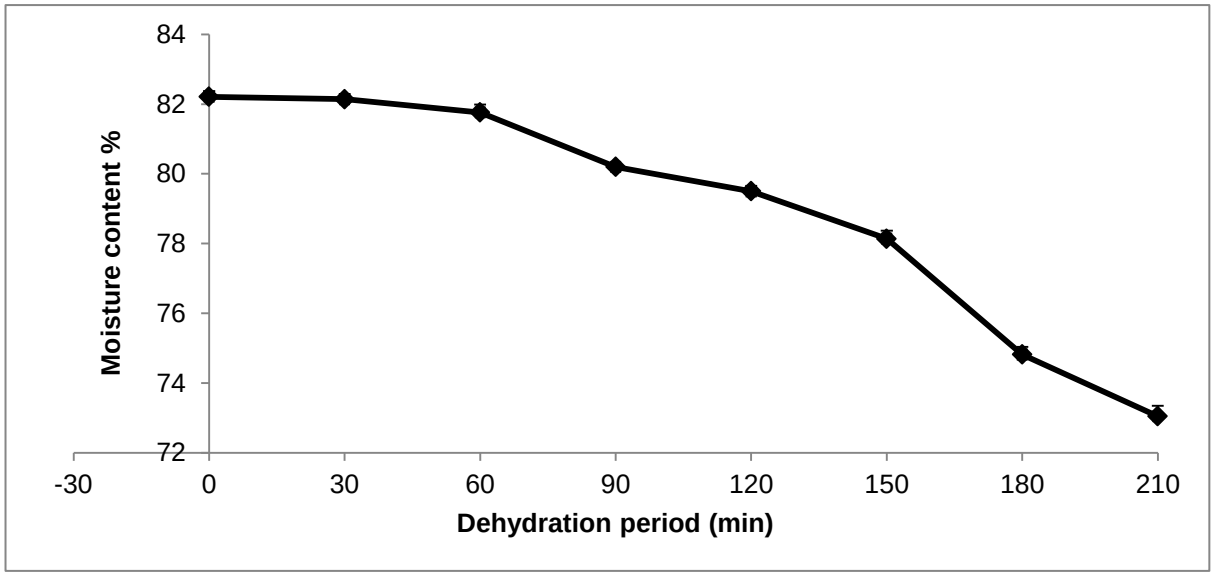

Figure 61. Effect dehydration period on moisture content of precultured encapsulated somatic embryos (LSD = 1.06).

5.2.3.3 Cryopreservation of encapsulated somatic embryos.

5.2.3.3.1 Objective

To evaluate the ability of ESEs and SEs (non encapsulated) for cryopreservation in LN using encapsulation–dehydration protocols. Different protocols were applied in order to investigate the optimal procedure suitable for storage of ESEs in LN.

5.2.3.3.2 Materials and methods

ESEs were precultured in liquid basal MS medium containing 0.75 M sucrose for 7 days followed by dehydration for 120 mins as described in section (5.2.3.2.2). The dehydrated capsules were placed in 2 mL sterile cryovials and subjected to various cryopreservation protocols as follows:

Protocol 1: SEs (non encapsulated) + direct immersion in LN 1h.

Protocol 2: ESEs (no preculture) + direct immersion in LN for1h.

Protocol 3 : Precultured ESEs with 0.75 M sucrose for 7 days at 5 ℃ + dehydration + LN for1h.

Protocol 4: Precultured ESEs with 0.75 M sucrose for 7 days at 5℃ + dehydration + cryo-freezing to -20 ^{o}C + L N for 1h.

Protocol 5: Precultured ESE's with 0.75 M sucrose for 7 days at 5℃ + dehydration+ cryo-freezing to -40 ^{o}C + LN for 1h.

Following cryopreservation the capsules were rapidly thawed in a water bath at 40 ºC for 3 min and then transferred to liquid basal MS medium supplementing with 1.2 M sucrose for 10 min and then cultivated on germination medium (basal MS medium supplemented with 1 mg L^{-1} Kinetin , 1 mg L^{-1} IBA and 3% sucrose). Cultures were incubated at 22.5 ˚C under 16 photoperiod at 80 µmol m^{-2} s^{-1}. Survival of cryopreserved encapsulated somatic embryos was calculated after one month.

5.2.3.3.3 Results

The results of this experiment showed that none of the treatments with ESEs or SEs survived the freezing temperature of LN. Also it was observed that SEs on all protocols died (turned a white colour) after a few days of cultivation on germination medium .

5.3 Disscusion

The work described in the chapter investigated the cryopreservation of cauliflower in three forms – as embryogenic callus, somatic embryos and encapsulated somatic embryos as well as cold storage of embryogenic callus tissues.

Long term storage of RDECT by preculture-dehydration technique

The preculture-dehydration techniques that were used in this study for cryopreservation of cauliflower RDECT appeared as successful survival (regrowth) of RDECT after freezing in LN for 1 h using fairly simple protocols which are easy to apply. It was observed that the growth of RDECT decreased after thaw-freezing process. This reduction might be due to the number of cells that have been killed or injured through the freeze-thaw process however, subsequently rapid multiplication of RDECT was observed. Similar results were reported by Ulrich et al., (1982) on callus cultures of date palm. However, the best embryogenic potential of cauliflower RDECT was observed when cold preculture with 0.7 5M sucrose for 7 days at 5℃ or 10 ℃ and dehydrated for 90 min followed by immersion directly in LN was applied. Somatic embryos that appeared on explants of callus tissue developed to the globular stage but did not progress to other developmental stages. However, positive effects were achieved with survival and regeneration after cryopreservation of RDECT when preconditioning of plants such as cold acclimation and sucrose preculture were used. This might be due to the maintenance of cell viability during dehydration and cryopreservation which achieved by the accumulation of sucrose inside tissue as sucrose can help in the stabilization of membranes (Crowe et al., 1984, Oliver et al., 1998, Crowe et al., 1988). Also the pre-treatments may have improved desiccation tolerance and led to a reduction in the free cell water content, preventing or restricting intracellular ice crystal formation (Vicient and Martínez,

1998). A progressive dehydration process was used in this study consisting of preculture and dehydration to dewater the RDECT. In preculture, the use of a high sucrose concentration for 7 days at 5 ℃ was most efficient. However, increased concentration of soluble sugars is a typical characteristic in cold-acclimated plants (Levitt, 1972). Soluble sugars are known to have an essential role in cryoprotectant, osmoprotection and mobilization of other protective substances during cryopreservation (Hincha, 1990, Hitmi et al., 1999) since sucrose could penetrate the cell wall, but not the plasma membrane In case of cells are frozen, sucrose is concentrated in the cell wall space and protects protoplasts from freeze-induced dehydration. It can form a buffer layer between the protoplast and the cell wall in order to protect the outer surface of the plasma membrane (Tao and Li, 1986).

Simliarly, preculture with 0. 75 M sucrose at 4 C for 7 days was used successfully with *Dioscorea bulbifera* L. calli (Ming-Hua and Sen-Rong, 2010). However, the use of non-freezing temperatures at 5 ℃ was reported in conifer to enhance immature embryos to develop ultra low temperature tolerance when used for 4 weeks without cryoprotectant and this maintained embryogenecity after cryopreservation (Kong and Aderkas, 2011). Moreover, shoot tips of Shih (*Artemisia herba-alba Asso*.) achieved high survival after preculturing with different sucrose concentrations at low temperature 4 C (Sharaf et al., 2012).

However, preculture-dehydration technique was used for cryopreservation non encapsulated embryogenic callus tissue of sweet potato (*Ipomoea batatas*) with retaining its competence to produce SEs (Blakesley et al., 1996), embryogenic calli *of Quercus robur* L. (Chmielarz et al., 2005), embryogenic tissues of wild cherry (*Prunus avium* L.) (Grenier-de March et al., 2005) and embryogenic tissues of *Picea omorika* (Serbian spruce) (Hazubska-Przybyl et al., 2010). Moreover, the

cryostorage technique utilizing cryoprotectants and partial dehydration has been applied for embryogenic axes of *Pisum sativum* (Mycock et al., 1995).

Despite producing SEs from some protocols used in a current study, other protocols led to a loss of the potential for producing SEs from RDECT. However, the loss of post-thaw viability and subsequent embryogenic competence was reported for embryogenic callus of cassava that was cryopreserved by vitrification technique. This might be due to the sequential two step dehydration that caused by osmotic dehydration followed by freeze dehydration which might be resulted in disorginazation of cells of cryopreserved embryogenic callus tissue and led to loss the viability (Danso and Ford-Lloyd, 2011). This observation is similar to the findings in this current work.

Also, the loss or decrease of embryogenic competence was reported in sweet potato (*Ipomoea batatas*) and attributed to the loss of a large percentage of tissue with embryogenic potential after rapid freezing in LN. This tissue still proliferated and produced friable callus after cryopreservation using the encapsulation-dehydration technique (Blakesley et al., 1995).

Moreover, Gonzalez-Benito et al., (2009) referred to a decrease in the embryogenic capacity of cryopreserved grapevine cells whilst in contrast Wang et al., (2002) reported that embryogenic tissue which were stored in LN might have a positive effect on its embryogenic competence because elimination of non-embryonic cells occur. While survival of cells that can develop into somatic embryos could achieve. Such recalcitrance to tissue culture or the cryopreservation process can be found in many species and successful cryopreservation has not been guaranteed for all plants (Katkov, 2012). This lack of reproducibly using the protocols reported in this

study suggests that further improvements in protocols are required to develop their efficiency for conservation. The formation of SEs using some protocols however, can be considered an important and promising finding since another modifications on these protocols could lead to improvement in cauliflower RDECT preservation for long term storage using preculture dehydration techniques. These could include different non–freezing temperatures such as 1, 2, 3, or 4 ℃ through preculture treatment or another slow-freezing temperature treatment such at -5, -10 and -15 as the use of -20 and -40 before immersion in LN (-196) did not develop the cryopreservation capacity of cauliflower RDECT.

Short term storage of RDECT by cold storage at 5℃

In the current study, cauliflower RDECT showed a high capacity for successful cold storage at 5 ℃ without an intervening subculture and the embryogenic potential of RDECT stayed the same after three months of cold storage in darkness. This is an important finding as it can lead to a reduction in the cost of maintenance of cauliflower RDECT by reducing the number of subcultures which also reduces the risk of contamination which can happen during subculturing. However, a slow growth of cauliflower RDECT under current storage conditions was observed, thus it is suggested that a lower temperature e.g. 1 or 2 ^{o}C rather than 5 ^{o}C might be required to prolong the storage duration. The storage of cultures at low temperature (2-8 ℃) has been extensively used for other species (Bajaj, 1991). Callus cultures from various species have been stored for 4 to 6 months without subcultures at low temperatures or under a mineral oil overlay (to reduce dehydration) (Augereau et al., 1986). Callus cultures of tobacco were stored for two or four months at 4°C depending on callus strain (Hiraoka and Kodama, 1984) and embryogenic calli of grape stored at 10°C can survive and maintain its ability for embryogenesis after 360

days of storage (Moriguchi et al., 1988). Also, cold storage at 4 ^{o}C was reported to effectively maintain viability of garlic (*Allium sativum*) shoot cultures after 16 months without subculture (El-Gizawy and Ford-Lloyd, 1987) and shoot tips of *Lolium multiflorum* Lam for a period over of three years (Dale, 1980) and cold storage at 5℃ in dark was reported for shoot cultures of *Trifolium repens L*. cv. Grasslands Huia for 10 months with 100 percent survival and rapid subsequent propagation (Bhojwani, 1981). The storage of *Eucalyptus grandis* encapsulated axillary bud for 3 months at low temperature and light intensities were obtained without loss of viability (Watt et al., 2000). The storage at 6°C was reported by Westcott et al., (1977) for nodal cultures of potato which maintained their ability to produce new growth after twelve months.

Embryogenic potential such as the capacity to form mature SEs which can develop into plants can be maintained through cold storage (George et al., 2008). Although, RDECT tissue used in the experiments here was more than two years old the formation of SEs was still in the same range as freshly derived callus. This result is in accordance with Yasuda et al. (1985) who reported the ability of embryogenic callus tissue to produce somatic embryos after two years of subculture in *Coffea arabica.* Sarkar (2009) stated that when subculture of embryogenic callus tissue occurs, the continued formation of somatic embryos can obtained through it. Embrygenic calli of triploid bermudagrass (*Cynodon transvaalensis* x *C. dactylon*) can be propagated continuously for at least 2 years with a high regeneration ability to re-establish the culture system (Lu et al., 2006). In contrast, the decrease in regeneration capacity of embryogenic lines of *Pinus nigra* was mentioned by Salajova and Salaj (2005) when long periods of culture were used and some lines lost their regeneration ability.

Long term storage of somatic embryos by encapsulation–dehydration technique

In the current study, although ESEs of cauliflower appeared to have ahigh capability for preculture and partial dehydration as a high survival rate was achieved after these two processes, they could not survive immersion in LN in all protocols tested. This is probably due to that the damaging ice formation in the somatic embryos as the water content of ESEs was high (79.5%) even after dehydration, leading to lethal ice crystal formation during freezing. However, in zygotic embryos, the maturation process usually includes some dehydration which can reduce the metabolism and normally leads to the quiescent or dormant state. In somatic embryos by contrast the tolerance to dehydration appears to be very limited as the slowdown to dormancy does not occur (Vonarnold et al., 2002). It is possible that the quiescent phase resembling true seeds could be provided through desiccation of somatic embryos (Senaratna, 1992) or by synthetic upregulation of drought protection mechanism. The preparation of embryos for cryopreservation is important and requires dehydration of the SEs to optimal water content but the preparation of SEs tissue in a dehydration process is a perquisite. Therefore, a partial dehydration using a cryoprotectant could be the answer (Mycock et al., 1995) such as development of SEs on media enriched with a high concentration of sucrose (Dumet et al., 1993b). The effects of sucrose concentration through its accumulation in the tissue (Dereuddre et al., 1991b) or the artificial seed (bead) (González-Arnao et al., 1996) on the rate of dehydration may be important and might affect tissue cryo-tolerance (Fang et al., 2004). It has been observed that desiccated somatic embryos of white spruce survived freezing treatment at higher frequency compared to non desiccated embryos (Attree et al., 1995). In the present study, the positive effect that was observed on survival of ESEs after dehydration might be due to the effect

of stress-promoting morphogenesis especially in the apical meristem as the differentiation of axillary cotyledonary meristems were noticed. A similar effect for starvation and or dehydration was reported on germination of chestnut somatic embryos (Corredoira et al., 2008). Also, the high survival of pea somatic embryos was reported when pretreated with glycerol and sucrose followed by partial dehydration (Mycock et al., 1995). Moreover, pre-culture with high concentrations of sucrose increased freezing tolerance of alfalfa (*Medicago sativa*) (Senaranta et al., 1989) and oil palm somatic embryos (Dumet et al., 1993d). However, the whitening a tissue of SEs (death) after LN exposure which was distinguished in the current study was also reported previously in copreserved shoot-tip clumps of banana (*Musa spp.*) (Panis et al., 1996) and shoot–tips of *Citrus australasica* (Reed, 2008).

The encapsulation–dehydration method has been widely used for many plant species (Shatnawi et al., 1999, Shibli, 2000). This technique was used successfully for cryopreservation SEs of black iris (Shibli, 2000), 'Nabali' olive (*Olea europea* L.) (Shibli and Al-Juboory, 2000), cocoa (*Theobroma cacao* L.) (Fang et al., 2004), several genotypes of citrus (González-Arnao et al., 2003), *Vitis vinifera* cultivars Brachetto and Muller-Thurgau (Miaja et al., 2004) and *Picea sitchensis* (Sitka spruce) (Gale et al., 2008) also, the recalcitrance to cryopreservation of cauliflower encapsulated microshoots was recently reported by Rihan (2013). Different technical approaches still need to be discovered to improve the efficiency of cryopreservation techniques for recalcitrant plants like cauliflower .

5.4 Conclusion

The current study described for the first time a simple and reliable *in vitro* cryopreservation and cold storage of cauliflower RDECT and SEs. The improvement of recovery after cryopreservation can be achieved using a preculture–dehydration technique and it was observed that the preculture of RDECT on MS medium enriched with 0.75 M sucrose at 5 or 10℃ for 7 days and dehydration treatment for 90 min reduced moisture content resulted in the best regrowth and survival of RDECT. Furthermore, this protocol subsequently led to the formation of somatic embryos after 40 days from culture on liquid SIM. Further experiments are needed to improve the cryopreservation capacity of cauliflower RDECT using preculture–dehydration but it appears to be a promising technology for the conservation of RDECT. The cauliflower somatic embryos using encapsulation–dehydration technique also requires more investigation to improve cryopreservation of cauliflower artificial seed via somatic embryos. Short term storage at 5 ^{o}C was applied successfully and RDECT was stored for three months at 5℃ without loss of its capacity for somatic embryo production. This can be considered an ideal approach for storage of RDECT as it is simple and easily applied and does not require sophisticated equipment or protocols. Within the time frame of this project longer storage times at 5 ^{o}C were not able to be tested, but there is clearly potential for much longer storage periods but these needs to be evaluated further.

Chapter six

Morphological comparison of plantlets derived by somatic embryogenesis with seedlings of original seedlot

6.1 Introduction

6.1.1 Zygotic and somatic embryogenesis

The processes of zygotic and somatic embryogenesis result in similar outcomes but follow very different developmental pathways. Zygotic embryogenesis commences after gamete fusion to produce a single cell zygote and ends with the production of the mature embryo whereas somatic embryogenesis originates from a single or a small collection of somatic cells. Integrated events can be distinguished through embryo development including mitosis, initiation of polarity, cellular differentiation, the formation of complex metabolites (including hormones) and storage of reserve materials (Dodeman et al., 1997). Zygotic and somatic embryos are bipolar structures and essentially consist of an axis with shoot and root apices. Ontogenetically, both embryo types undergo several developmental stages characterized by their morphology and termed globe, heart, torpedo and cotyledonary, however in contrast to zygotic embryos, somatic embryos can develop in the absence of vascular connections with the original plant (Zimmerman, 1993). Four distinct stages can be involved in propagating plants by somatic embryogenesis consisting of initiation of embryonic tissues, maturation of somatic embryos, germination and acclimation of somatic plants (Klimaszewska et al., 2007). Since somatic embryos are formed without any fertilization, they are genetically identical to the cells from which they are derived and thereby the parent plant from which those cells derived (Deo et al., 2010). Plants derived from these somatic embryos should therefore have the growth and development characteristics of the plant from which they were derived (Li et al., 1998) and appear phenotypically uniform (Vasil, 1982). Such uniformity (sometimes called stability) has been previously confirmed for somatic embryos of cauliflower (Leroy et al., 2000). Furthermore in broccoli, Yang et

al., (2010) reported that somatic embryos had the same DNA content as their mother plants and somatic embryo derived synthetic seeds of *Cucumis sativus* showed genetic stability and similarity to mother plants as proved by using RAPD markers (Tabassum et al., 2010). The genetic stability of somatic plantlets for several plant species was also confirmed in several studies (Mo et al., 1989, Ikeda et al., 2006, Thakur et al., 1999, Fernandes et al., 2011, Valladares et al., 2006). Despite such evidence there is always a doubt associated with somatic embryos that they may carry DNA mutations accumulated during disorganized cell proliferation during the callus phase of *in-vitro* culture and some of these can be manifested as somatic phenotypic mutations. It is important therefore with any new somatic embryogenesis protocol to check offspring.

6.1.2 Acclimation of somatic embryos

Commercially, the ultimate success of micropropagation depends on the ability to transfer plants out of culture on a large-scale with high survival rates at low cost (Chandra et al., 2010). Plantlets or shoots that have been grown *in vitro* have been exposed to a unique micro-environment that is selected to achieve minimal stress and optimum conditions for plant propagation and plantlets have grown within culture vessels under aseptic conditions in an atmosphere with high level of humidity and low level of light on medium containing ample sugar and nutrients to provide heterotrophic growth (Hazarika, 2003). These special conditions during *in vitro* culture can lead to the formation of plantlets of abnormal morphology, physiology and anatomy. When these plantlets are transferred to *in vivo* conditions they may easily be impaired by sudden changes in environments (Pospóšilová et al., 1999). In contrast the glasshouse and field have substantially septic, lower relative humidity and higher light level environments that are stressful to regenerated plants which

have been produced *in vitro* culture conditions (Hazarika, 2003). High rates of loss of plants can occur due to low humidity and when water is limiting owing to low hydraulic conductivity of root and root-stem connection in plants from in-*vitro* conditions (Fila et al., 1998). It has been found that there were deficient vascular connections between the root system and the stem (Grout and Aston, 1977) and the roots that form *in vitro* culture are often non-functional and therefore these roots can be eliminated at the time of acclimation to induce new functional rooting *in vivo* (Debergh and Maene, 1981). In a study on leaves of cauliflower, it was observed that there were reduced quantities of epicuticular wax on plantlets *in vitro* versus on seedlings or acclimated plantlets produced from culture (Grout, 1975, Grout and Aston, 1977) and this might be lead to excessive wilting and eventual death of the propagated plants on their removal from culture conditions (Grout, 1975). Acclimation of propagated plantlets can overcome these problems with a gradual lowering in air humidity (Lavanya et al., 2009). During acclimation to *in vivo* conditions, leaf thickness generally increases, leaf mesophyll progresses in differentiation into palisade and spongy parenchyma, the stomatal shape changes from circular to an elliptical one and stomatal density decreases and one of the most important physiological changes is effective stomatal regulation of transpiration leading to stabilization of water status (Pospóšilová et al., 1999). Therefore, plantlets of tissue culture origin should be slowly acclimated or hardened off in order to survive the transition from culture tube to glasshouse or field conditions (Wetzstein and Sommer, 1982). Often the physiological abnormalities of tissue culture plantlets can be repaired after transfer to *in vivo* (Pospóšilová et al., 1999). In the acclimation process, somatic plantlets can be covered with glass beakers for one week. After that, the acclimated plantlets are exposed to glasshouse conditions by removing the

cover partially at first and then full removal. Following *in vitro* culture, a gradual decrease in relative humidity for regenerated plantlets is required to acclimate to glasshouse conditions prior to planting in the field (Jain and Gupta, 2005). If the *in vivo* transplantation is successful, an increase in plantlet growth can be achieved (Pospóšilová et al., 1999).

6.1.3 Climate and soil

In cauliflower, the vegetative and reproductive phases, including curding, are affected by temperature. Cauliflower plants can grow at an average temperature of 5-8 ℃ to 25-28 ℃, and grow well in a cool moist climate. The optimum temperature for growth of young plants is around 23 ℃ and for the later stages is 17-20 ℃ (Board, 2004). The heads do not develop well in hot weather. In regions with no frosts, planting might be made at any time of the year when water can be provided for the growing the crop, whilst in regions where hard freezes can occur, well-hardened plants might be planted out as early in the spring as the ground is prepared or as soon as the danger of hard frosts is over (Din et al., 2007). In dry and hot weather, plants might fail to form desirable heads as these conditions lead to premature heading (bolting) and/ or buttoning (Mujeeb-ur-Rahman et al., 2007). Time of curd initiation after the end of the juvenile phase depends on temperature; delayed curd initiation and increased final number of leaves occur at higher temperatures (Booij, 1990b). Thus, temperature can be is considered as a major factor in curd initiation (Salter, 1960; Sadik, 1967) and under high temperature, some varieties stay at the vegetative stage (Haine, 1959; Booij, 1987). It was shown that it is important to select a suitable variety according to climatic conditions for commercial cultivation as each variety or genotype has different requirements for curd initiation. Variation in cauliflower responses were observed in response to photoperiod and reduction in

total irradiance delays curd initiation under warm conditions, while increased irradiance can present as partial substitute for low temperature in accelerating curd initiation (Hand, 1988; Sadik, 1967). Cauliflower plants grow well on sandy loam to clay loam soils which are rich in nutrients and well-drained. The ideal soil for growing cauliflower is a fairly deep loam. Cauliflower is sensitive to high acidity and the optimum soil pH is 5.5-6.5 (Board, 2004).

6.1.4 Physiological disorders

In Brassicas/Cole crops, physiological disorders can be defined as abnormalities in stem and leaf morphology, color, or both which are not caused by infectious diseases or insects. The occurrence of these abnormalities occurs due to environmental stress, nutritional deficiencies or excesses on the plant (Masarirambi et al., 2011). Cauliflower suffers from a number of physiological disorders that manifest in various types of disease syndromes. Some might be owing to environmental, organic and inorganic nutritional imbalance and some might be genetically controlled (Board, 2004). Physiological disorders are divided into groups: genetic predisposition (blindness, buttoning, head splitting and bolting); nutrient imbalances (internal tip burn) and watering disorders (head splitting, buttoning) (Norman, 1992).

For example rolled leaves are one of the symptoms of boron deficiency in the cabbage family (Chandler, 1940, Chandler, 1944) and sometimes leaves of boron deficient plants are yellow and blistered (Masarirambi et al., 2011). However, the young leaves of cauliflower grown in growth chambers suffer from tip burn as a symptom of calcium deficiency (Wiebe and Krug, 1974). Tip necrosis of young expanding leaves surrounding the enlarging curd cause lower product quality and, in

severe cases, might discolor the curd owing to secondary pathogen infection and lead to a loss in marketable heads (Warner et al., 1981).

The disorder of riceyness is characterized by a curd surface which is loose and has a velvety appearance with small white flower buds forming at the curding stage. Heavy applications of nitrogen and humidity can lead to riceyness (Board, 2004) and riceyness also occurs when the growing temperature is lower than the optimum temperature for curd development. But, when the growing temperature is higher than the optimum temperature for curd development, fuzzy head bearing developed bracteoles can be formed, this might be as a result of partial reversion of curd development to the vegetative phase. When the curd of cauliflower is exposed to temperature higher than inducing fuzzy heads, leafy heads occur in which green leaves grow through the surface of the curd. The development of these leaves is from axillary bracts of primary peduncles (Fujime and Okuda, 1996). Hollow stem is another disorder, the development of hollow stem and curd occurs in heavy fertilized soils especially with nitrogen (Board, 2004, Masarirambi et al., 2011).

Moreover, buttoning which is the development of a small curd in a young plant and fewer, less developed leaves can also sometimes occur in response to variable temperatures. Cauliflower can be considered a very sensitive crop and any check in its growth at some stage such as deficiency of nitrogen, transplanting of older seedlings, water stagnation in the field, planting an early type of cauliflowers under low temperature can cause buttoning. The appearance of Blindness is a disorder when damage to the growing point by low temperature, frost or insects occurs during an early stage of growth. In this case, plants grow without a terminal bud and they fail to produce curd. Due to accumulation of carbohydrate, the leaves of blind plants become thicker and leathery. Whiptail is another disorder in cauliflower, and the

deficiency of molybdenum particularly in acidic soils results in whiptail. In this disorder, normal leaf blade development fails and the formation of only strap like savoyed leaves are observed and in extreme cases, only the midrib will be developed. Whiptail is corrected by application of molybdenum and the liming of soils (Board, 2004).The production of healthy and strong cauliflower plantlets is perquisite to continued normal growth in field conditions. Thus, there is great interest to achieve this aim through developing a reliable procedure for acclimation of cauliflower SEs from which platelets are produced rapidly and to allow the development of SE generated plants in the field and to investigate the morphological characteristics of somatic and zygotic plants.

6.2 General materials and methods

6.2.1 Sowing the zygotic seeds

The zygotic seeds of cauliflower cv. White Cloud were planted in plastic trays (23x 37x 5.5 cm) which contained soil and compost (1:1) on 21 March of 2012 (the time of planting in the glasshouse and in the field were tested in the previous year 2011 during summer and winter time and the best times were applied in this experiment). The trays were kept in a glasshouse on the campus of Plymouth University. After germination, the seedlings were watered every two days (Fig.62 A). After one week, 50 seedlings were separated and transferred to big pots 13 x 12 cm to follow their growth (Fig.62 B). After forming four leaves, the survival rate for seedlings was recorded before transferring to the field (Fig.62C). Twenty five seedlings were chosen and transferred to the field; the survival rate in the field was recorded after a further month.

Figure 62. A) Plantlets of zygotic embryos through full germination. B) Transfer plantlets to pots with mixture of soil and compost. C) Growing plantlets with formation the true leaves.

6.2.2 Acclimatization procedure of SEs

Fifty germinated SEs were extracted from the germination medium (MS devoid of growth regulators). Rooted SEs (Fig.63 A) were carefully transferred out of the medium when plantlets were well developed with a normal shoot and root, their roots are washed with running tap water to remove culture media attached to the roots avoiding damaging the roots. In the first week of May, the plantlets were transferred to plastic containers (36 x 55.5 x 17 cm) containing a mixture of 1:1 (v/v) soil: compost (sterilized by autoclaving for 30 min). The mixture was well watered with tap water before culture. The acclimatization steps were applied at room temperature (25˚C). To maintain the humidity, the plantlets were covered with plastic pots for the first week (Fig.63 B). After that, they were gradually exposed to the room humidity; plastic pots were removed gradually when plants showed new growth. After two weeks of acclimatization, the plantlets grew vigorously (Fig.63 C). After three weeks, the survival rate for acclimated SEs was recorded and 25 plantlets were chosen and transferred to the field to follow their growth. The survival rate of plantlets in the field soil was calculated after one month of transferring to the field assessed as = (number of surviving plants/total number of plantlets X100).

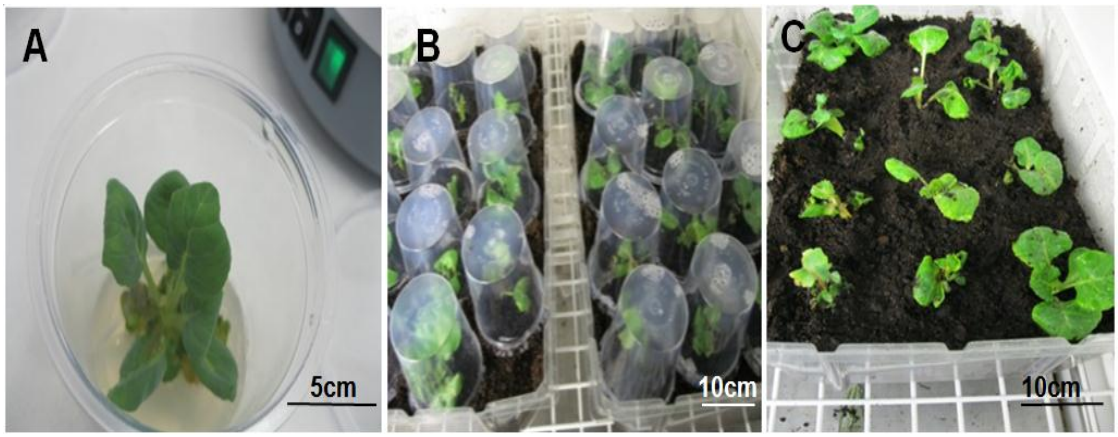

Figure 63. Plantlets of cauliflower somatic embryos through the acclimation process that included:

Plantlet of SE on semi solid germination medium. B) Plantlets of SEs covered with plastic pots. C) Plantlets growing well after raising plastic pots with new leaves formed through acclimation period.

6.2.3 Plant morphology, fresh weight measurements

Various phenotypic characteristics of both zygotic plants and somatic embryo-derived plants were recorded at harvest time. A ruler was used to measure the height of each plant, from the point on the stem at the soil surface to the point of the apical meristem, (to the nearest centimeter) and the diameter of curd was also recorded. Leaf number was counted as all fully expanded leaves on the stem at harvest. Harvested curds of plants were weighed using a balance (Oxford- Model A 2204) to determine the fresh weight.

6.2.4 Statistical analysis

All data were statistically analysed using Minitab 16 using one-way ANOVA. Significant differences between treatments were determined using least significant differences (L.S.D) at the 0.05 level. The experimental design was a randomized block. All graphs were plotted using Microsoft Excel 2010. All data were pre-tested for normality distribution using Minitab Basic Statistics which showed the data were normally distributed and did not require transformation.

6.3 Field experiment for year 2012

6.3.1 Objective

To establish an efficient system for plant recovery of somatic embryo derived plant to the field and plant production phase and to compare SEs plants with zygotic plants.

6.3.2 Materials and methods

The experiment was carried out during 2012 under field conditions at Plymouth University, UK. The survived platelets that produced from acclimated somatic embryos and seedlings of zygotic seeds were transferred at the same time and stage of growth (plantlets with four leaves) to outside the glass house for one week before transplanting in the well prepared field. Trays of plantlets were irrigated so that the plantlets could be easily taken out from the beds without damage to the roots. Fifty plantlets, 25 produced from somatic embryos and 25 produced from seed were planted on 30 May 2012 in the field. Plantlets were spaced at 30 cm between plants and 45 cm between rows. After one week, 2.0 ml of the MS salt solution was added as fertilizer. Another fertilizer (N/P/K, 10: 10: 27, Phostrogen Ltd. UK) was added after one month and plants were all treated against pests when required. Plantlets were watered by a regular watering schedule. Hoeing, weeding and earthing up operations were applied continuously through growth stages as well as blanching through curds maturation stage. The mean air temperature and maximum and minimum temperatures during the curd maturation period are presented in Figure 64 (Data were obtained from the Plymouth University meterological station).

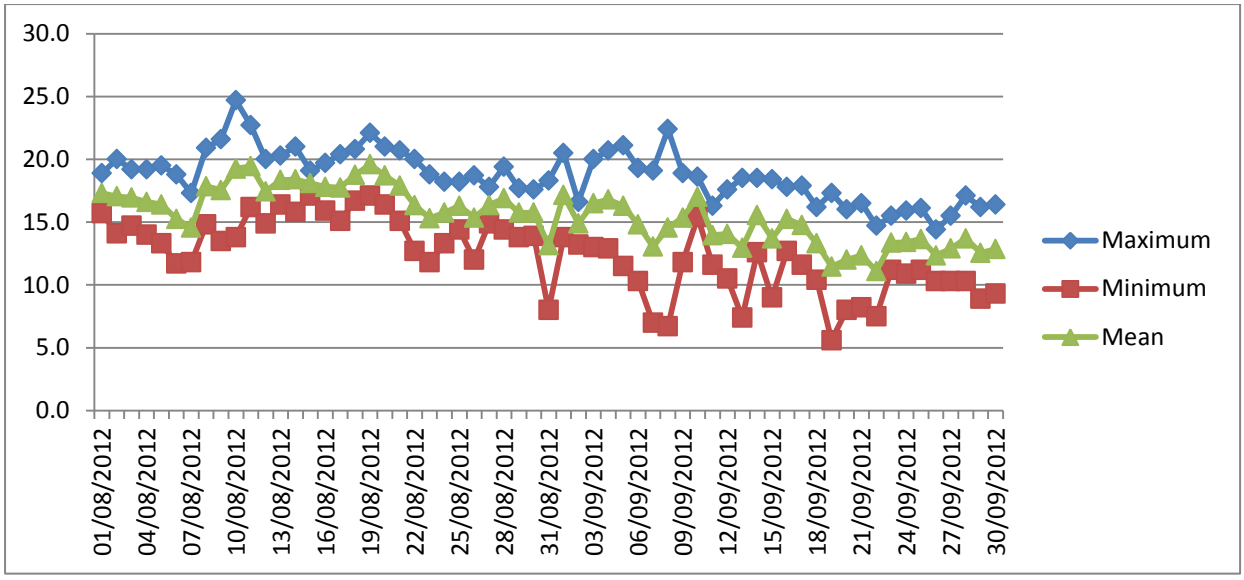

Figure 64. Maximum, minimum and mean air temperatures during curd maturation period (through August and September in year 2012).

6.3.3 Results

6.3.3.1 Survival rate of acclimated SEs and planted of zygotic seeds

The results revealed that a 100% survival rate was achieved for seedlings produced from zygotic seeds when assessed through two periods, after forming four leaves (before transferring to the field) and after one month of transferring to the field. In contrast an 80% survival rate was achieved for plantlets from somatic embryos after three weeks of acclimation plantlets with four true leaves) but the subsequent transplanting survival rate was 96% one month after transferring of acclimated plantlets to the field. As shown in Figure 65, the development of plantlets that were produced from SEs was uniform and the plants were normal.

Figure 65. A) Plantlets of zygotic embryos with four leaves (before transferring to the field. B) Plantlets of somatic embryos with four leaves after three weeks of acclimation (before transferring to the field.

6.3.3.2 Plantlet development and yield.

The results presented here were obtained from 17 plants of zygotic embryos and 13 plants of somatic embryos as the rest plants were lost before collecting data (snail infestation). Visual observation of plants in the field through vegetative and flowering stages indicated that the growth of somatic embryos plants were normal and morphologically similar to that of zygotic plants. The leaves had a similar shape and structure for both sets plants. During the first two weeks of growth in the field, leaves of somatic plantlets appeared to have a more greenish color, but after that, the colour was similar for all plants (Fig. 66 & 67). Moreover, the curds had the same white color in both and it was compact and well formed (Fig 68). The measurement of leaves number at harvest time revealed that no significant differences between somatic and zygotic plants (P = 0.173). The plants differed in height with zygotic plants being taller (Table.4). In terms of the days from planting to curd initiation, the results showed that curding in plants of both types started at the same time and they required a mean of 60 days. Following curd initiation, it was clear that the plants of zygotic embryos needed less mean of days for curd maturation to harvest with a mean of 63 days while somatic plants needed a mean of 91 days. However, the

highest mean of curd fresh weight and diameter was achieved from zygotic plants. Little variation between plants of somatic embryos in terms of size of curd formation (assessed as diameter and fresh weight) was observed.

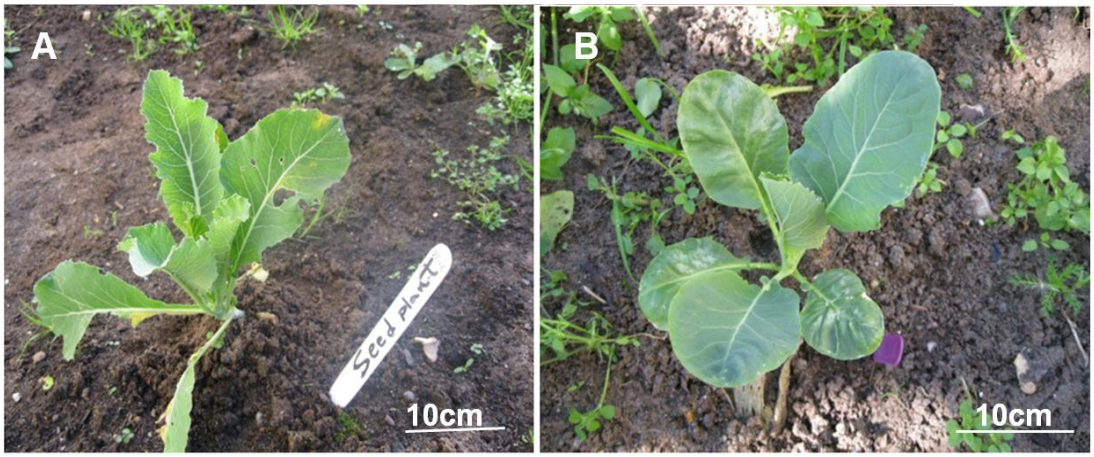

Figure 66. A) Plantlet of zygotic embryo after one month of transfer to the field. B) Plantlete of somatic embryo after one month of transfer to the field.

Figure 67. Plantlets of somatic and zygotic embryos growing in the field after two months of transferring (start of flowering).

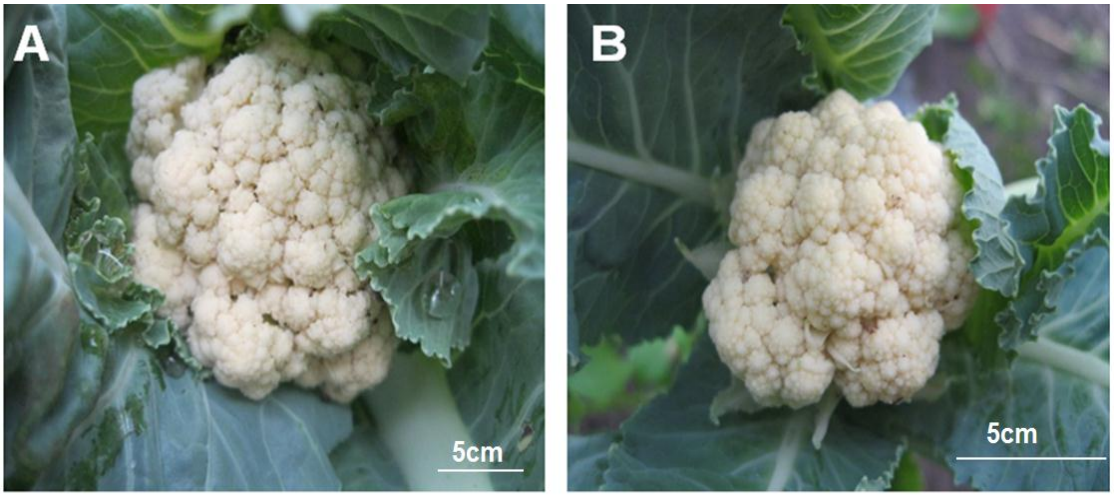

Figure 68. Plants of both zygotic and somatic embryos with white curd formation. A) Plant of zygotic embryo. B) Plant of somatic embryo.

Table 4: The effect of plant type on some characteristics of cauliflower plantlets growing in a field.

Plant type	Leaf number	Plant height (cm)	Curd diameter (cm)	Curd fresh weight (g)	Days from planting to curd initiation	Days from curd initiation to harvest	Mean
Zygotic plant	25.8 a	28.5 a	9.1 a	94.1 a	60 a	63 a	46.75
Somatic plant	23.4 a	20.7 b	6.3 b	28.8 b	60 a	91 b	38.36
Mean	24.6	24.6	7.7	61.4	60	77	42.55
P value	0.173	0.002	< 0.001	< 0.001	1.000	< 0.001	

6.4 Field experiment for year 2013

6.4.1 Objective

To confirm the results that were obtained from the field experiment of 2012 using more plantlets produced from both zygotic and somatic embryos.

6.4.2 Materials and methods

During March 2013, the zygotic seeds of cauliflower cv. White Cloud were planted in plastic trays (see section 6.2.1). In terms of plantlets of somatic embryos, more than 200 plantlets were prepared for this experiment (Fig. 69). The acclimation of these plantlets took place in May 2013 using the same procedure reported in section (6.2.2).

Figure 69. Plantlets produced from somatic embryos during March 2013 inside the incubator (five plantlets in each pot).

6.4.3 Results

The results of this experiment revealed that seedlings produced from zygotic embryos were successfully produced in the glasshouse but unfortunately the plantlets produced from somatic embryos could not continue their growth through acclimation and died after a few days of acclimation (Fig. 70 A&B). It was observed

that the leaves of platelets inside incubator were smaller than had been observed before and hooking of most leaves as well as collapse of middle area of leaf tissue (Fig. 71 A&B) had occurred. This led to no somatic plants being weaned for the field experiment.

An investigation to know the reason for this failure was undertaken. The temperature was the same as usual inside incubator (22.5 ˚C) and light intensity was 25 µmol m^{-2} s^{-1}. To investigate, different treatments were conducted as described below.

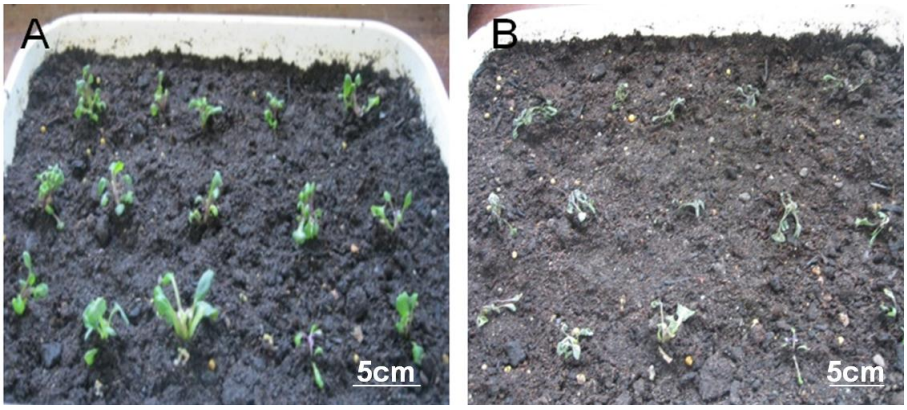

Figure 70. A) Plantlets of SEs directly after acclimation. B) Plantlets of SEs after one week of acclimation.

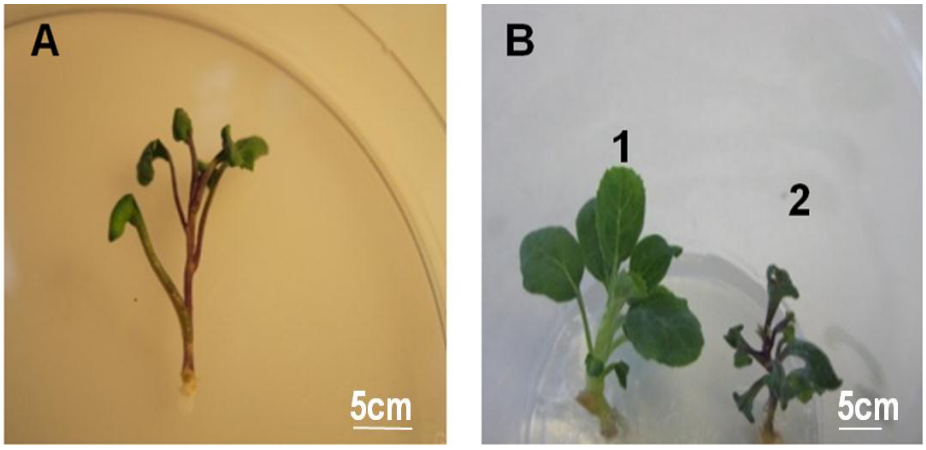

Figure 71. A) Plantlet of somatic embryo with some physiological disorders. B) 1. Intact plantlet of somatic embryos. 2. Plantlet of somatic embryo appears with physiological disorders.

6.5 Effect of different factors on growth of somatic embryos inside the incubator.

6.5.1 Objective

To investigate the reasons that caused hooking and collapse of leaf tissue of SEs inside incubator that led to the loss these plantlets though the acclimation process.

6.5.2 Materials and methods

Two groups of treatments were used:-

The effect of MS and agar source:

Four treatments were applied including:-

Original MS (Sigma- Aidrich) + original agar which was phyto-agar 7 g L^{-1} (Duchefa Biochemic).

Original MS + new agar which is agar gel 3.5 gL^{-1} (Sigma- Aidrich)

New MS (Duchefa Biochemic) + original agar

New MS + new agar

Five explants were cultured in each pot which contained 30 mL of medium; five replicates were used for each treatment. The pots were kept in the original incubator. Observations were recorded after three weeks.

The effect of : -

MS strength: - Full and half old MS were used.

Activated charcoal: - Two treatments including two concentrations of 1 and 2 mg L^{-1} were used.

Kinetin concentration: - Three concentrations were used (2, 4 and 6 mg L^{-1}).

Calcium chloride ($CaCl_2$): - Four additional concentrations to that of MS were added (50, 75, 100 and 125 mg L^{-1}).

H_2BO_3 : - Three additional concentration to that of MS were added (0.25, 0.5, 1 mg L^{-1}).

Three explants were placed in each pot containing 30 mL of medium. Three replicates were used for each treatment. Two replicates were kept in the old incubator and the third replicate in another incubator. The observation was recorded after three weeks.

Light intensity: - Three levels of light intensity were used (25, 50 and 100 µmol m^{-2} s^{-1}). Three explants were placed in each pot containing 30 mL of original MS media. Three replicates were used for each level. After that, cultures were kept in another new incubator for three weeks.

6.5.3 Results

After 21 days of incubation, the same symptoms appeared on plants. It was found that there was no effect of light intensity and the temperature was the same inside the incubator. Furthermore, MS salt strength, MS and agar source, the use of additional concentration of some elements like calcium and boron or hormones like Kinetin concentration as well as the use of activated charcoal did not appear to have any positive effect on the plants. This means that the cause of the deformations is a mystery and further investigations are required to determine the reasons behind these physiological disorders.

6.6 Discussion

Field experiment for year 2012

A simple, reproducible and reliable procedure was accomplished for acclimation and development of plantlets that were produced from cauliflower SEs and zygotic embryos. To our knowledge there are no previous reports in the literature on the effectiveness acclimation of plantlets produced from cauliflower SEs. In the work

reported here, a high rate of survival was achieved from acclimated cauliflower SEs and from zygotic embryos through weaning and germination in a glasshouse and after transferring to the field. Thus, this indicates that this procedure can be considered applicable for large scale production of plantlets. This work parallels that of other species where a high rate of survival was established from cork oak plantlets acclimated from multiple-lines of somatic embryogenesis (Pintos et al., 2010) and from loblolly pine (*Pinus taeda* L.) somatic embryos after planted in a field (Pullman et al., 2003). Recently, an 80% survival rate was established from hardened somatic embryos of gherkin (*Cucumis anguria* L.) (Thiruvengadam et al., 2013). Previously, it was reported that acclimation and the transfer to the *in vivo* environment is a difficult step that frequently causes the micropropagation system to fail (Litz and Litz, 1999, Menéndez-Yuffá et al., 2010, Paul et al., 2011). Hernandez et al., (2003) transferred 703 platelets of *Quercus suber* to *in vivo* conditions and only 33 survived in the field six months later. Also, a plantlet regeneration rate lower than 1.2% was obtained from *Populus nigra* microspore cultures (Deutsch et al., 2004). However, good progress was achieved for acclimation of cauliflower somatic plantlets in the present work. In terms of growth ability of plantlets in the field, somatic plantlets showed normal growth in comparison to plants from natural seed. Different parameters were used to examine and compare the quality of plantlets produced from somatic embryos to that of zygotic embryos such as morphology and fresh weight. Morphologically, it was observed that cauliflower plants produced from SEs were uniform and similar to plants produced from seed. This similarity in phenotype was also reported in other plants such as in sweet potato (*Ipomoea batatas* Poir) (Schultheis et al., 1994), in cacao (*Theobroma cacao* L.) (Li et al., 1998), in Indian *Solanum surattense* (Swamy et al., 2005), in napier grass (*Pennisetum purpureum*

Schum) (Haydu and Vasil, 1981), in paradise tree (*Melia azedarach)* (Vila et al., 2003). Also, all gladiolus (*Gladiolus hort.*) (Stefaniak, 1994) and *Gymnema sylvestre* (Retz) R. Br. Ex Roemer and Schultes (Ahmed et al., 2009) plantlets produced via somatic embryogenesis did not differ from their parental clones.

In the current work, the observation that somatic plantlets had more greenish leaves than zygotic plants in the first two weeks of growing in the field might be due to accumulation of chlorophyll in the leaf tissues during incubation in culture room. It was observed that the plants of SEs had normal leaf development and the total number of formed leaves does not differ significantly at harvest time compared to plants from zygotic seed. However it was clear that zygotic plants were taller than somatic plants. Similarly, Yaacob et al., (2012) found that *in vivo* plants of African blue lily (*Agapanthus praecox* ssp. *minimus*) were taller than somatic plants that derived from *in vitro* culture. Whearse, Webster et al., (1990) have observed that final height, growth rates, shoot and root morphology and frost hardiness of interior spruce (*Picea glauca* (Moench) Voss x *Piceae ngelmannii* Parry) somatic plantlets were similar to those of seedlings plants. However, this difference in plantlet growth might be due to the development of the aerial and root systems of plantlets grown in soil are better than those of plantlets grown *in vitro.* Thus the root systems are more extensive have numerous fine roots and more branched leading to suggest that strong nutrient uptake and growth potential can occur (Etienne- Barry et al., 2002).

In the current experiment, after two months of planting in the field, normal cauliflower curd initiation was observed from both somatic plants and seed but somatic plants required more time for maturation with a longer period from initiation until harvest date. In accordance with the present investigation, it was reported that the plants produced from somatic embryos of *Brassica juncea* (L.) Czern & Coss are normal in

flowered and their pod setting (Kirti and Chopra, 1989). Also, the same results was reported in corn (Vasil et al., 1984) and Indian` *Solanum surattense* somatic plants (Swamy et al., 2005).

Small late curds of cauliflower were reported previously by Crisp (1984) who conjectured that this might be due to a consequence of their slow growth rather than their late initiation. At the end of the harvesting period of a crop, curd size is often reduced and this is more marked if environmental conditions reduce the mean curd size and lengthen the time to maturity. It is suggested that this is in accordance with the results reported here. Moreover, the prolonged period for somatic plantlets to follow their growth until harvest date was also reported by Schultheis et al., (1994) who found that the SEs plants of sweet potato require more time for roots to bulk or size than other propagules used including plants of zygotic seed. However, it was found from the present work that fresh weight and diameter of curd was less in somatic plants than zygotic plants. This reduction in yield was also reported in sweet potato where the production of larger sized storage roots (bigger than 6 cm in diameter) was lower from plants that produced from somatic embryos (Schultheis et al., 1994). Moreover, the reduction in fresh weight and size of curds in plants of cauliflower in the current study might be due to mollusc infection (by snails) which affected both somatic and zygotic plants.

Field experiment for year 2013

It was a pity that the somatic plants were not able to be weaned for the second growth season and unfortunately this could not be repeated in a third year due to time constraints. The rapidly organized experiment to try to isolate the source of the morphological problem was not successful either. The use of different source of agar and MS, different MS strength, additional concentration of some macro elements

such as calcium or micro elements such as boron (as rolled leaves are one of boron deficiency symptoms) had no effect on the abnormal development of the somatic plants. However, we suggest that activated charcoal may have a positive effect as mentioned previously by Thomas (2008) who reported that AC had promoter effects on morphogenesis mainly owing to its irreversible adsorption of inhibitory compounds in the culture medium and substantially decreasing the toxic metabolites, brown exudate accumulation and phenolic exudation. But the results showed that there was no effect of AC in the medium. Also it was observed that the use of different concentration of Kin and different levels of light has no effect on this phenomenon. The cause of the problem under the experiment conditions remains unclear. It can be speculated that these symptoms it might be due to some other deficiency of nutrients in the MS but more research is required to investigate and determine the reasons that caused this phenomenon and this should also include an investigation of the gaseous environment.

6.7 Conclusion

Major progress was made in the area of the acclimation process, the survival rate and development of somatic plantlets in the field. Somatic plants showed normal growth relative to seed derived plantlets. It can be concluded that the regeneration through somatic embryos can maintain the morphological characteristics of the mother plant despite some differences in plant height. In terms of plant curd formation, the initiation of curd was at the same time in both zygotic and somatic plants but the size of the curd was bigger in zygotic plants and these curds needed fewer days for maturation.

Chapter seven: General Discussion

7.1 Somatic embryogenesis in cauliflower

Hybridization systems based on either self-incompatibility or male sterility of the parent lines are important for F1 hybrid seed production in *Brassica* crops. However development of F1 hybrids is costly because years of selfing ae required to achieve the stabilization of inbred parental lines and thereafter breeding line maintenance is labour–intensive (Bhalla and de Weerd, 1999). Alternative systems can be based on mass clonal propagation of elite phenotypes and the most effective of these is somatic embryogenesis but for cauliflower no reliable somatic embryogenesis system is reported in the literature. Therefore, in the research reported here, an efficient and reliable method for *in vitro* propagation of cauliflower via somatic embryogenesis was investigated and developed for artificial seed production. Two mechanisms can be followed to initiate somatic embryogenesis, either directly on explanted tissue or indirectly through the formation of unorganized tissues (callus). The protocol developed through the present investigation will be useful for large-scale regeneration from callus tissue of cauliflower. The propagation of plants can be obtained through somatic embryogenesis as an alternative to organogenesis (Maqsood et al., 2012). In somatic plant cells, the reactivation of cell division is a perquisite for dedifferentiation (Nagata et al., 1994) and also to establish the embryogenic competence (Dudits et al., 1995, Yeung, 1995).

The first step of somatic embryogenesis is the initiation of embryogenic cultures and this can usually be achieved by culturing the primary explant on medium provided with growth regulators, mainly auxin but also often cytokinin (Von Arnold et al.,2002). The results presented here showed that embryogenic callus tissue of cauliflower was affected by explant type, concentration of growth regulators and medium type. The explants of seedlings were used and among the tested explants it was found that

hypocotyls appeared to have a high ability for callus induction and proliferation on semi solid media supplied with 2,4-D and Kinetin which required a subculture of callus tissue onto fresh medium at 21 day intervals. In this respect, Metwali and Al-Maghrabi, (2012) also reported the effectiveness of using agar media supplemented with a combination of auxin and cytokinin (2,4-D and BAP) in inducing callus tissue from hypocotyls of cauliflower. In some species and some genotypes the embryogenic cultures are subculturd onto medium containing PGRs for a prolonged period and still retain their potential for producing mature somatic embryos that can form plants (Geroge et al., 2008). In the current proliferation system for caulifower, long term subculture was achieved with root–derived embryogenic callus tissue (RDECT) which was subcultured for over two years and still maintained multiplicative capacity and did not change morphological characters. In plant tissue culture, the retention of embryogenic callus for a long period is very useful as it can facilitate the year round availability of somatic embryos in a regenerable state at any time (Pola et al., 2009).

The availability of an *in vitro* system that provides normal development of an embryo maintained in a physical and chemical environment different from inside the ovule, can lead to successful embryo culture (Slesak and Przywara, 2003). In the current study, the optimization of the embryogenic callus culture system on both semi-solid and liquid medium was described, and following initiation, embryogenic tissue was transferred to both temporary and continuous immersion in agitated liquid medium systems (bioreactors) for somatic embryo induction. An efficient propagation and mass production of somatic embryos was achieved in continuous immersion in an agitated liquid medium system and this system was superior to temporary immersion this might be due to the higher uptake and utilization of water and mineral nutrients

that are required for development. The explants are prevented from “drowning” (insufficient oxygen) in the liquid by the continuous rocking motion of the platform (shaker) (Metwali and Al-Maghrabi, 2012). The positive effect of agitated liquid medium in the development of propagated cauliflower explants was reported previously by Kieffer et al., (1995) and Kieffer et al., (2001) and recently by Metwali and Al-Maghrabi, (2012) and Rihan, (2013) but all of these systems used cauliflower curd explants. It appears that cauliflower is equally responsive in culture in many explant forms.

Yang and Zhang (2010) referred to somatic embryogenesis as a unique developmental process where somatic cells undergo restructuring to create embryogenic cells. After that, these cells can go through a series of biochemical and morphological changes that lead to somatic or non-zygotic embryo formation which have the ability for plant regeneration. However, somatic embryos can be distinguished by the main morphological characteristic of bipolarity and the absence of tissue connection with the explant vascular tissue (Falco et al., 1996, Gatica- Arias et al., 2007). In this somatic-to-embryogenic transition, cells can dedifferentiate and cell division cycles can be activated. This means that the cells have to reorganize their physiology, metabolism and gene expression patterns (Feher et al., 2003).

Under the present work conditions, several experiments were conducted to optimize the efficient proctocol for somatic embryogenesis in cauliflower. These experiments can be summarised as follows:

The optimization of size of embryogenic callus tissue. The size can be considered an important factor for initiation of somatic embryos and the size class of 600-1000

µm exhibited more induction and proliferation for somatic embryos when 90 sec blending duration of the callus was used.

The explant type was tested and it was found that root-derived callus explants were more inductive for regeneration through somatic embryogenesis. This is the first report that the SEs in cauliflower has been produced from root-derived callus explants. SEs were recently reported as being produced directly from hypocotyls and indirectly from leaf explants by Siong et al., (2011) and directly from immature zygotic explants by (Pavlovic et al., 2012).

Plant growth regulators, especially IAA and Kin and their concentration were confirmed as an important requirement for induction, development and maturation of cauliflower SEs. This was in agreement with previous studies on cauliflower somatic embryos (Pareek and Chandra, 1978, Deane et al., 1997) and the use of 2, 4-D and Kin as a combination was reported to form SEs in cauliflower (Siong et al., 2011). It was demonstrated in the study reported here that the appearance of some abnormalities such as SEs with three or four cotyledons occurred. Harrison and Von Aderkas, (2004) referred to phenotypic variation such as cotyledon number and showed that it could be induced by exogenous addition of growth regulators in the medium or altering hormone metabolism. These observations are in accordance with the results reported here since the SIM that was used included exogenous hormones.

Carbohydrates are required in plant cell, tissue or organ culture in order to satisfy energy demands (Amiri and Kazemitabar, 2011). These compounds are essential as the photosynthetic activity of *in vitro* grown tissues is usually reduced. Also, carbohydrates are necessary in media as osmotic agents. Therefore, carbohydrates often have a great potential effect on the physiology, growth and differentiation of

cells (Gibson, 2000). In the present work, two types of carbohydrate (sucrose and mannitol) with different concentrations were tested in the SEs production system and a significant effect was observed with sucrose at 2%. This finding is in contrast with what has been reported by Slesak and Przywara, (2003) in *Brassica napus* L. who found that SEs were produced on media including 6% sucrose and 12% maltose. Also sucrose at 6% was reported by Gerdakaneh et al., (2009) for somatic embryogenesis in strawberry (*Fragaria* × *ananassa* Duch.). These results demonstrate species specific sensitivity which can only be resolved empirically.

Both development and maturation of cauliflower SEs that was achieved in the present work was on the same somatic induction medium. This is useful because the use of one medium for induction and proliferation process can save time and energy as well as reduce cost. It was reported that germination of SEs can be obtained on medium devoid of hormone (Pliego-Alfaro and Murashige, 1988) and this was confirmed in the current study.

Importantly the work reported here is the first describing the full culture conditions which are necessary to produce secondary somatic embryos in cauliflower. It was demonstriated that somatic embryos can be an excellent source for production SSEs in cauliflower. The potential of embryogenic cultures to undergo repetitive somatic embryogenesis has made the development of propagation using somatic embryogenesis highly desirable (Baker and Wetzstein, 1995). Repeated cycles of culturing showed that some species can retain the embryogenicity for a long period (Raemakers et al., 1995). It was found in the current study that the presence of AC in the medium used for induction of SSEs has a negative effect because most of SSEs that formed were abnormal. However, the formation of embryos with fused cotyledons can result from interference with polar transport of auxin (in early globular

embryos as it is essential for the establishment of bilateral symmetry during plant embryogenesis) which causes a failure in the transition from axial to bilateral symmetry (Liu et al., 1993). The abnormality in somatic embryos on medium containing AC was also observed in cultures of carrot *(Daucus carota*) (Pan and Staden, 2001). On the other hand, it was demonstrated in the present work that SSEs of cauliflower produced from medium supplied with AC germinated well when transferred on medium containing IAA. This beneficial effect for AC on germination of SEs has also been reported in several studies (Chee and Tricoli, 1988, Bohanec et al., 2010).

7.2 Somatic embryos for artificial seed production

In the present investigation, development of a reliable protocol for the regeneration of plants from SEs using *in vitro* techniques was achieved for artificial seeds in cauliflower. In several commercially important crops, development technology of artificial seed production can be considered as an effective alternative method to F1 hybrid production however, in cross-pollinated species, the production of hybrid seed is a widespread practice. The use of a conventional breeding program for the creation of hybrids consumes much time and resources in obtaining and maintaining appropriate parental lines. Thus, the use of artificial seed to propagate elite genotypes without the need to generate parental lines is one of the possibilities to reduce costs in time and money (Desai et al., 1997) and makes storage and transportation easier (Ravi and Anand, 2012). Recently in cauliflower, attempts have been made to produce artificial seeds using micro-shoots of cauliflower from curd explants (Rihan et al., (2012) and Siong et al., (2012). To our knowledge, the study reported here is the first report on the mass production of artificial seed using somatic embryos in cauliflower.

The ability of using cauliflower SEs in artificial seed production was confirmed in the present work with the optimization of an ideal artificial endosperm. It is thought that the current investigation will have crucial effects on mass production of cauliflower artificial seed from SEs with low cost and reduced time as the production of artificial seed starting with callus induction and SEs formation reaching to encapsulation and artificial seed formation needed just 103 days. The SE encapsulation system is a promising procedure as the artificial seed matrix consists of SEs and calcium alginate containing essential nutritional components, plant hormones, a carbon source and antimicrobial agents. The alginate bead also protects SEs from mechanical damage (Tabassum et al., 2010). In the present investigation, it was found that the incorporation of additives into the encapsulating gel, especially PGRs, was more beneficial in enhancing the survival rate of somatic embryos. As such it was found that the enhancement of germination from encapsulated propagules requires optimization of the growth regulators in the culture medium instead of in the capsule gel. Cytokinin and auxin (Kin and IBA) were shown to have an effect on the germination of cauliflower encapsulated SEs. The positive effect of hormones added to culture medium on germination of encapsulated SEs was also reported in grape (*Vitis vinifera* L.) (Das et al., 2006), pedunculate oak (*Quercus robur* L.) (Prewein and Wilhelm, 2003) and mango (*Mangifera indica* L.) (Ara et al., 1999). Also, the effect of hormones in culture medium on conversion of encapsulated SEs was reported in *Artemisia vulgaris* L. with a high germination percent (90%) (Sudarshana et al., 2013). In terms of the current work, further research is still needed to develop the rate of germination using other concentrations of hormones or using another type of exogenous hormone in culture medium since the use of Kin and IBA led to the appearance of callogenesis and shoot formation from encapsulated SEs. Also the

incorporation of other hormones and additives such as MS and carbohydrates in the artificial endosperm (capsule gel) is prequisite to test.

7.3 Cryopreservation of embryogenic tissue and somatic embryos

The regeneration of plants through somatic embryogenesis has a crucial advantage as the embryogenic tissue has the potential to be cryogenically stored in liquid nitrogen (Kartha, 1985). In the present work, two approaches were used for storage of cauliflower RDECT, the first one was long term storage and involved the use of a preculture–dehydration techniques for cryopreservation in LN at -196℃. A high survival of RDECT was investigated for all protocols that were developed in this study and the embryogenic potential was observed from several protocols with few numbers of somatic embryos at the globular stage of development. This formation of SEs from cryopreserved tissue of cauliflower makes the production of mature SEs a potential improvement of this technique in the near future. This technique was used to preserve embryogenic callus tissue as was reported previously for other plant species (Blakesley et al., 1996, Grenier-de March et al., 2005, Chmielarz et al., 2005, Hazubska-Przybyl et al., 2010). Other researchers have also reported that embryogenic callus tissue can be cryopreserved successfully using an encapsulation-dehydration technique (Mandal et al., 2009, Bhatti et al., 1997, Blakesley et al., 1995). The second approach was a short term storage at 5℃ and cauliflower RDECT retained good embryogenic capacity when stored at this temperature for three months in the dark. This is in agreement with Jain, (2011) who found that at low temperature (0-5°C) the growth rate and the number of subcultures on fresh culture media can be reduced without influencing the genetic stability of cultures. Also, Vasil, (1982) referred to the proliferation potential for embryonic cell-lines that can be maintained for a long period in culture and it often has ability to

give a rise to a normal and uniform population. Clearly non-frozen cold storage can be used to maintain cultured plant cells as an alternative approach (Reed, 1991, Reed, 1993) and for certain species where it is not appropriate to be preserved in freezing temperatures this is essential (Hiraoka and Kodama, 1984). A positive effect of cold storage of cauliflower RDECT in the current investigation was achieved and this means that the cost of the subculturing process can be reduced and the loss of cultures through contamination that might be occur through subsequent sub-culture can be reduced.

The encapsulation-dehydration technique is one of the cryogenic procedures that can be used to avoid the toxic effect of cryoprotectants such as PVS (plant vitrification solution) and it is easy to handle (Tsai et al., 2009). Various explants such as somatic embryos, shoot tips and cell suspensions for a wide range of plant resources can be preserved using this technique (Wang et al., 2005, Yamazaki et al., 2009, Ming-Hua and Sen-Rong, 2010). In the present study, the lack of response of somatic embryos for cryopreservation was in accordance with Engelmann (1997) who referred that the complex tissues such as well organized somatic embryos and shoot tips which do not often appear to respond to cryopreservation using slow freezing.

7.4 Morphology studies for somatic and zygotic plantlets

The appropriate acclimation procedure leading to the production of whole plants with normal morphology and physiology which survive the transfer to the glasshouse is essential for any proposed *in vitro* system (Grout and Crisp, 1977). Therefore, an efficient acclimation procedure for cauliflower somatic and zygotic embryos was investigated in the current study leading to the production of healthy plantlets with high rate of survival after transferring to the field. Recently, in Brassica plants, SEs

of kohlrabi (*Brassica oleracea* var. *gongylodes*) were reported to be successfully acclimated in the greenhouse with a survival rate of 72.5% (Ćosić et al., 2013). Furthermore successful acclimation and establishment of plantlets was achevied for SEs of other plant species (Devaraju and Reddy, 2013, Mathews and Wetzstein, 1993, Perán-Quesada et al., 2004).

In the present work, morphological similarity was recorded between somatic and zygotic plantlets. Many researchers have stated that SEs plants can grow in a similar way to those derived from true seed (George et al., 2008) and they have similarity in morphology, biochemistry and physiology (Kitto and Janick, 1982). In spite of these similarties, several differences were observed in the current study in terms of the size of cauliflower curds which were smaller in SEs plantlets and took a longer time for ripening. In contrast to our investigation, plantlets of *Coffea arabica* L. that were derived from SEs were more vigorous than seedlings plantlets based on higher leaf number, leaf area and dry weight of aerial organs, which was attributed to the large diameter of roots, also somatic plantlets were more precocious than seedling plantlets and produced coffee beans 1 year earlier than seedlings (Menéndez-Yuffá et al., 2010). When such enhanced vigour occurs it is often attributed to the suppression or elimination of systemic non-lethal pathogens such as viruses.

7.5 Proposed future work

It was demonstrated in the current study that cauliflower primary SEs have the ability to produce secondary SEs on MS medium devoid of growth regulators. More experiments could be conducted to improve this capability of cauliflower primary SEs through the investigation of exogenous hormones effects in culture medium. This would lead to a higher efficiency of SE production.

It would be of interest to determine suitable exogenous hormones and other additives such as sucrose and MS strength that can be added to the artificial endosperm of artificial seed or in the culture medium to improve the germination capability of artificial seeds.

A fuller investigation of the propagation capability of artificial seed under greenhouse and field conditions is needed.

More research is needed to optimize and improve the embryogenic competence of cauliflower RDECT using preculture – dehydration technique as successful survival of RDECT after cryopreservation in LN was proven with formation of SEs from cryopreserved RDECT. The most important factor that needs to be optimized is the preparation phase of callus tissues towards dehydration (especially by sucrose and cold treatments). Researches should move toward standardizing and simplifying the method. Also, the prolonged period for cold storage of RDECT (more than three months) requires testing at more cold storage temperatures and for longer periods of time. Moreover, the development ability of somatic embryos for cryopreservation through improving encapsulation-dehydration technique or by using another technique is required.

It was noticed that the acclimation procedure that was used in the present study has a good impact on survival rates of SEs. Therefore, it might be useful to apply this procedure in further work to produce more SEs plantlets for field experiments to investigate the characteristics of SEs plantlets through to flowering and seed set. Also a fuller investigation of genetic stability of plantlets derived from SEs is required.

It will be important to investigate the reasons that led to the appearance of physiological disorders on plantlets of SEs inside the incubator that resulted in the loss ofmost of them through the acclimation period. A positive response could be

obtained by an increase or decrease in some macro and micro element concentrations in the culture medium.

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Appendices

Embryogenic callus induction and proliferation in cauliflower (*Brassica oleracea var. botrytis*)

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Introduction

A callus is a mass of unorganized cells, which upon transfer to suitable medium is capable of giving rise to shoot-buds and somatic embryos, which then form complete plants. Callus can be classified into two kinds embryogenic and non-embryogenic and embryogenic callus is competent to develop into the various stages of somatic embryos. The out-breeding nature of cauliflower creates difficulties in the achievement of purebred lines. Also, the high cost of hybrid seed and relative unavailability have inhibited the popularization of F1 hybrids in many Brassica species. Somatic embryogenesis can be used to shorten the long sexual breeding cycle and eventually can solve the problem of F1 seed availability. Cauliflower is reportedly recalcitrant to somatic embryogenesis .

Aim

•The aim of the present study was to investigate the induction and proliferation of embryogenic callus in Cauliflower.

Objectives

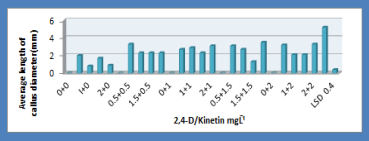
• To induce callus using different explants including cotyledon, hypocotyls and root using various concentration of 2,4-D and Kinetin.

•To study the ability of embryogenic callus to produce somatic embryos.

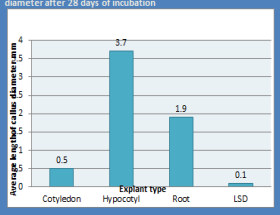
•To investigate the effect of type of culture system on callus proliferation through subsequent culture.

Results

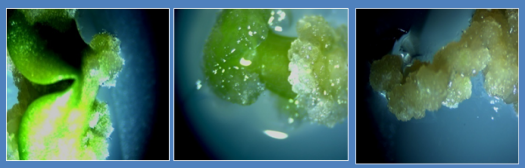
Callus induction



The effect of different levels of 2,4-D and Kinetin on average callus diameter after 28 days of incubation



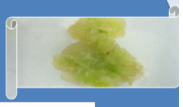
The effect of various explants (Cotyledon, Hypocotyls, and Root) on values length of callus diameter.

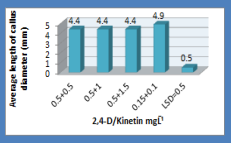


Cotyledon Hypocotyl Root

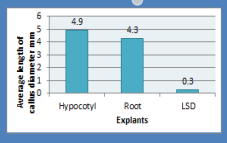
Callus proliferation

1-On semi solid media



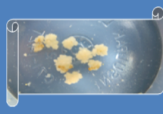


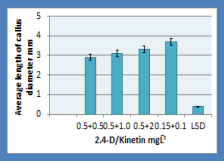
The effect of exogenous hormone combinations of 2,4-D and Kinetin on callus diameter during subsequent culture on semi solid media.



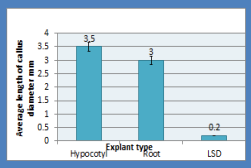
The effect of explant origin type on subsequent callus growth.

2-In liquid medium

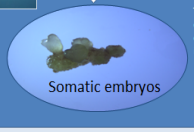




The effect of exogenous hormone combinations of 2, 4-D and Kinetin on callus diameter during subsequent culture in liquid media



The effect of explant origin type on subsequent callus growth in liquid media



Somatic embryos

Conclusion

It can be concluded that both the plant growth regulators used and the explant type had an effect on both callus initiation and subsequent callus culture. The optimum concentration for embryogenic callus induction and subsequent culture was 2,4-D at 0.15 mgL^{-1} and Kinetin at 0.1 mgL^{-1}. The results showed that hypocotyl explants were superior during callus induction and subsequent culture (on both semi solid and liquid medium). In comparison to the other explants used, it was bright green and friable which is indicative of embryogenic potential. It was observed that the growth of embryogenic callus during subsequent culture was greater on semi solid medium but callus tissue also demonstrated a very embryogenic behaviour during subsequent culture in liquid medium. The best period for subsequent sub-culture was 21 days.

The effect of explants type on callus induction in cauliflower

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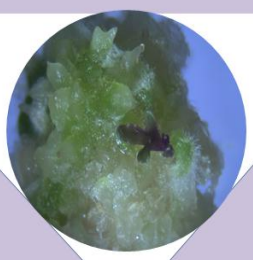
Introduction

A callus is a mass of unorganized cells, which upon transfer to suitable medium is capable of giving rise to shoot-buds and somatic embryos, which then form complete plants.

Plants can be propagated from unorganized callus tissues derived from various explants induced by exogenous growth regulator. Somatic embryos can be obtained indirectly through callus formation. The use of different explants such as root, hypocotyl and cotyledon are applied for callus production in Brassica seedlings. The explants are cultured on media having comparatively high auxin (2,4-D, 2,4-dichlorophenoxyacetic acid) and form an unorganized mass of cells, called callus. The callus can be further sub-cultured and multiplied.

The Objective

To produce somatic embryos because callus cultures on solid media or in suspension cultures can form embryo-like structures called somatic embryos, which on conversion (germination) produce complete plants.



Results

Results

*An efficient callus production was achieved in this study

*The explants were swollen after 7 days of culture and the callus appeared within 14 days, after 28 days the average callus diameter varied greatly among the explants

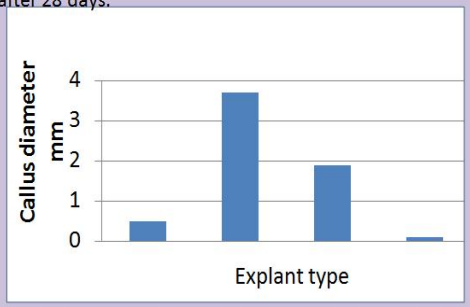
a) * A bright green friable putatively embryogenic callus proliferated on the cut edges of the hypocotyl explants

*Hypocotyl explants were showed statistically better capacity for callus induction in terms of callus diameter, color and texture

*It was noted that the root explants appeared to show significant variation in values of callus diameter and produced a more yellow friable callus

*Cotyledon explants were the least response for callus production.

Effect of various explants on values length of callus diameter after 28 days.



Conclusion

Could conclude from this experiment that the explants type had an effect on different callus growth The results showed that hypocotyls explants was superior to the other explants, it had the highest value of calli diameter (3.7mm), bright green and friable callus was produced .Whereas root derived callus was yellow friable.

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The Production of Cauliflower Microshoots using Curd Meristematic Tissues and Hypocotyl–derived Callus.

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Introduction

In vitro shoot propagation from Brassica seedling explants provides a plentiful supply of responsive explants from many plant structures including cotyledons, seedling roots, hypocotyls, stem sections, petals and leaf tissue. In particular shoot formation with high proliferation frequencies have been achieved from hypocotyl explants of commercial cauliflower genotypes. In vitro propagation favours the culture of pre-existing meristems because of their superior genetic stability and the cauliflower curd has a great advantage, since it contains millions of meristems forming an ideal basic material for tissue culture applications. This study aimed to investigate the ability of using both meristimatic and hypocotyls tissues for cauliflower microshoot production.

Materials and Methods

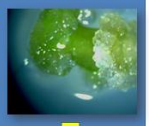
The use of meristematic layer

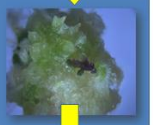
The use of hypocotyl derived callus

Plant preservative mixture + PGRs

PGRs (1mgL-1 2,4-D + 1.5 mgL-1Kinetin

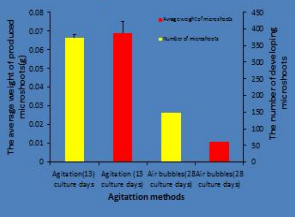
PGRS (0.5 mgL-1Kinetin)



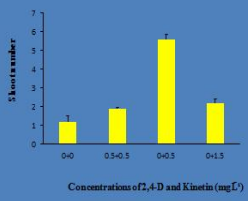


Results

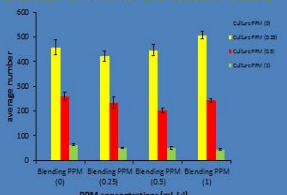
The effect of agitation method



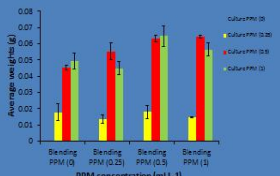
The effect of (2, 4-D and Kinetin) on shoot formation after 28 days (LSD=0.48



The effect of PPM in the culture media



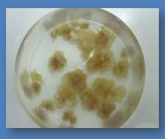
The effect of PPM used with culture medium and blending medium on the number of developing microshoots after 21 culture days (LSD = 7.601)



The effect of PPM used with culture medium and blending medium on the average weight of microshoots after 21 culture days (LSD = 0.0066)

The effect of 1mg L-1 2,4-D and 1.5 mg L-1 Kinetin on callus induction.

The effect of 1mg L-1 2,4-D and 1.5 mg L-1 Kinetin on callus proliferation



The effect of 0.5 mg L-1Kinetin.

(0) ml L-1 PPM

(0.25) ml L-1 PMM

0.5 ml L-1 PPM

1 ml L-1 PPM

Conclusion

Cauliflower microshoots were optimised using both curd meristimatic layer (without callus formation) and hypocotyls–derived callus. The use of the meristematic layer protocol was more effective in terms of the number of microshoots and the reduction of the time required to the end point of shoot production. Kinetin was an essential plant growth regulator for shoot induction. Further studies are required to investigate the effect of microshoot resources on the quality of microshoots and their growth capacity in subsequent stages towards in-vivo plant production.

Acknowledgment

Hail Rihan gratefully acknowledges a PhD grant from the University of Damascus, Syria. Magda Al Shamari gratefully acknowledges a PhD grant from the Ministry for Higher Education, Baghdad, Iraq. The authors gratefully acknowledge the technical staff (Angela Harrop and Michele Kieman) at Plymouth university .

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The use of somatic embryogenesis in artificial seed production in cauliflower *(Brassica oleraceae var. botrytis)*.

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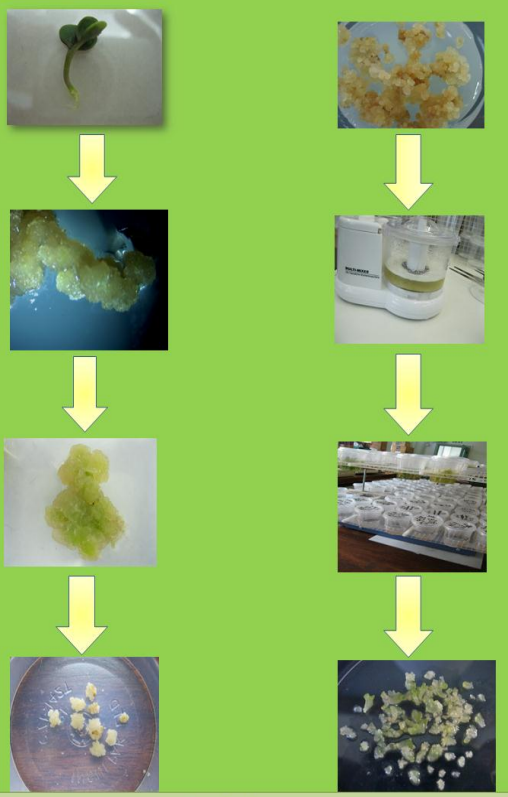
Introduction

Somatic embryogenesis is the developmental pathway by which bipolar structures that are similar to zygotic embryos are derived from somatic cells without gamete fusion. In this asexual embryogenesis method external bipolar structure is normally formed without connection to the plant or callus vascular system, and it has root and shoot poles. Basically, both embryos undergo the same stages of development and go through globular, heart shaped, torpedo, cotyledonary and mature embryos morphologies. They can also germinate and produce new plantlets The word embryoid is frequently used when embryo-like structures appear in cultures with an appearance similar to zygotic or somatic embryos .

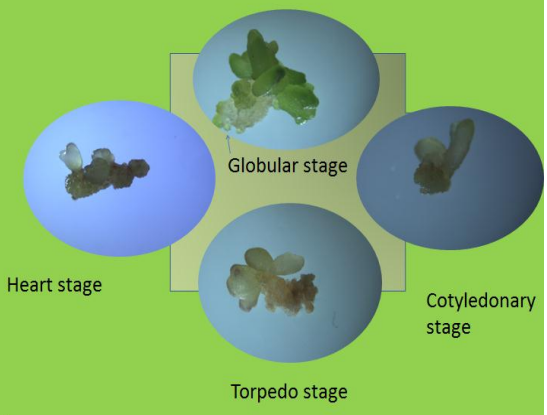
Materials and Methods

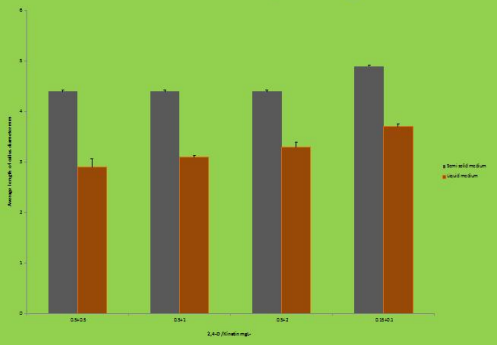
Callus induction

Somatic embryos formation



Results

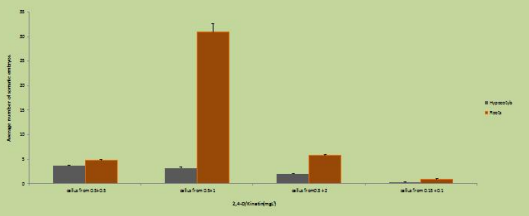




The effect of exogenous hormone combinations of 2,4-D and Kinetin on callus diameter during subsequent culture on semi solid medium(LSD=0.5) and in liquid medium(LSD=0.4).

Conclusion

A reliable method was developed for the high production of cauliflower somatic embryogenesis. Optimum embryogenic callus proliferation was obtained using friable callus derived from hypocotyls and root explants on semi solidified MS media. High production of somatic embryos were established on MS agitated liquid medium supplemented with 0.05 mgL^{-1} IAA (Indole -3-acetic acid), 0.5 mgL^{-1} kinetin and 20% sucrose after 20 days. The highest survival and conversion rate of 100%and 60% respectively was achieved after one month when somatic embryos cultivate on MS medium without hormones.



The interaction effect of embryogenic callus produced on four types of (callus induction medium)CIM and two type of explants in average number of somatic embryos produced on MS medium containing 0.5 mgL^{-1}Kinetin and 0.05mgL^{-1}IAA (LSD=6.23).

Primary and secondary somatic embryos in cauliflower *(Brassica oleraceae var. botrytis).*

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Introduction

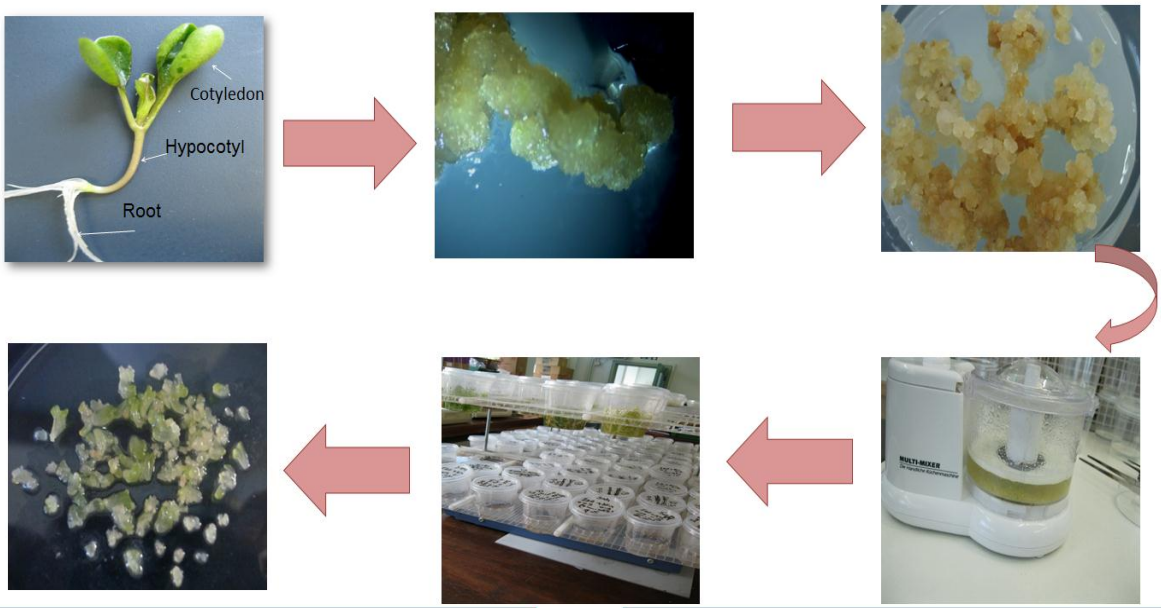
Somatic embryogenesis is a multi-step *in-vitro* regeneration process which starts with pro-embryogenic mass formation followed by somatic embryo formation, maturation, desiccation and plant proliferation. This method includes the development of embryos from somatic cells which often pass through stages morphologically similar to zygotic embryogenesis . The developmental stages of *in-vivo* embryogenesis can be reflected by somatic embryogenesis as they pass through globular,heart and torpedo shaped stagesThe use of *in-vitro* somatic embryogenesis is preferred over other *in-vitro* developmental processes such as organogenesis or axiliary bud propagation, since it can be used for micropropagation or genetic modification. The secondary somatic embryogenesis process is a special case of direct somatic embryogenesis. The emergence of secondary embryoids apparently originate from single epidermal cells of swollen hypocotyls. and from surface of cotyledons of primary somatic embryo. In this phnomenon a new somatic embryos can be created from somatic embryos.

The objective

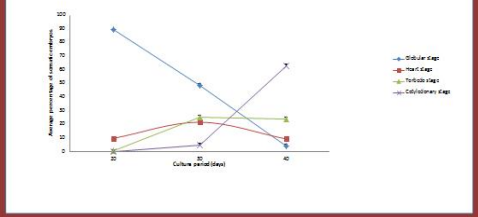
The objective of this study was to investigate the ability of ECT to produce primary somatic and secondary somatic embryos.

Results

The results revealed that the use of primary somatic embryos as explants leads to form secondary embryos on MS basal medium free of hormones but supplemented with activated charcoal(AC) or without . Secondary embryos were visible from hypocotyls of the primary SEs within 60 days of culture. It was observed that small mass of tissue proliferated from the tissue at hypocotyls of primary embryos, after that several Secondary embryos (SSEs) emerged. Different developmental stages of SSEs were noticed. primary somatic embryos on MS basal medium free of hormones and AC exhibited the best induction for secondary embryogenesis.



Effect of period culture on somatic induction medium on the percentage of somatic embryos at all developmental stages .(LSD for globular stage=6.05 ,for heart stage=7.34,for torpedo stage=8.98 and for cotyledonary stage=6.07).



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Conclusion

A reliable method can be followed to produce primary and secondary somatic embryos from explants of cauliflower. Following this breakthrough a protocol was developed for the mass production of somatic embryos using a bioreactor system. The optimization of culture conditions for induction, development, germination and conversion of primary somatic embryos to plantlets is described. The effect of exogenous growth regulators and sucrose concentration as well as the explants size and their source on embryo formation was evaluated and optimized.